Advances in CANCER RESEARCH

Volume 74

Edited by

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ACADEMIC PRESS
San Diego London Boston
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0065-230X/98 $25.00

Academic Press
a division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.apnet.com

Academic Press Limited
24-28 Oval Road, London NW1 7DX, UK
http://www.hbuk.co.uk/ap/

International Standard Book Number: 0-12-006674-2

PRINTED IN THE UNITED STATES OF AMERICA
98 99 00 01 02 03 BB 9 8 7 6 5 4 3 2 1

Contents

Contributors to Volume 74 vii

Key Effectors of Signal Transduction and G1 Progression
Martine F. Roussel

I. Introduction 1
II. The G1 Phase of the Mammalian Cell Cycle 3
III. Signal Transduction and G1 Progression 5
IV. The RAS/ERK Pathway and the Cell Cycle 5
V. RAS, D-Type Cyclins, and RB Connections 9
VI. Cycling with MYC 11
VII. Interplay between MYC and Cyclin D1 13
VIII. Signaling and Cell Cycle Roles of the SRC Family of Kinases 14
IX. Concluding Remarks 15
References 16

p53 in Tumor Progression: Life, Death, and Everything
Michael R. A. Mowat

I. Introduction 25
II. Biochemical Activities of p53 26
III. p53 and Cell Cycle Control 27
IV. p53 and Apoptosis 29
V. p53 and Tumor Progression 37
References 42

Signal Transduction through MAP Kinase Cascades
Timothy S. Lewis, Paul S. Shapiro, and Natalie G. Ahn

I. The MAP Kinase (MAPK) Module 49
II. Mammalian MAPK Pathways 50

Venema, J., van Hoffen, A., Karcagi, V., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. (1991). *Mol. Cell. Biol.* **11**, 4128–4134.
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Signal Transduction through MAP Kinase Cascades

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I. The MAP Kinase (MAPK) Module
II. Mammalian MAPK Pathways
 A. ERK1/2 and MEK1/2 Pathways
 B. Stress Activated Protein Kinase Pathways
III. Regulation of MAPK Pathways by Protein Phosphatases
 A. Dual Specificity Phosphatases
 B. Serine/Threonine Phosphatases
 C. Protein Tyrosine Phosphatases
IV. Cellular Substrates of MAP Kinases
 A. Protein Kinase Substrates for MAPKs
 B. Nuclear Transcription Factors
 C. Signaling Components
 D. Cytoskeletal Proteins
V. Responses to MAPK Pathways: Growth and Differentiation
 A. Regulation of Cell Growth and Transformation
 B. Regulation of Cell Differentiation and Development
VI. Yeast MAPK Pathways
 A. *Saccharomyces cerevisiae* (Budding Yeast)
 B. *Schizosaccharomyces pombe* (Fission Yeast)
VII. Intracellular Targeting and Spatial Regulation of MAPK Pathway Components
 A. Signaling Complexes
 B. Nuclear Translocation of MAPK and MEK
VIII. Future Directions
 References

I. THE MAP KINASE (MAPK) MODULE

The identification of MAP kinase pathways exemplifies the power of combining biochemical and genetic approaches to molecular problems. Components in these pathways were first discovered in genetic studies of the pheromone-regulated mating response in *Saccharomyces cerevisiae* as genes that showed homologies with protein kinases (Errede and Levin, 1993). Although these enzymes could be ranked genetically, evidence of their protein kinase activities and the realization that they functioned in the context of a

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kinase cascade awaited biochemical studies of growth regulation in mammalian cells.

As a result of earlier work establishing the importance of protein phosphorylation in cyclic nucleotide and calcium and phospholipid-regulated pathways, studies carried out during the 1970s focused on identifying kinases mediating growth factor and hormone action, particularly insulin. A key finding was the observation of growth factor-dependent phosphorylation of ribosomal protein S6 implicating factor-stimulated protein kinases in growth regulatory pathways. Combined with the discovery of tyrosine phosphorylation and receptor tyrosine kinases around 1980, protein kinase cascades in growth regulation were anticipated based on the paradigm of hormone regulated kinase cascades in the regulation of glycogen breakdown. Biochemical studies on hormone signaling in mammalian cell growth and oocyte maturation utilized strategies of cell stimulation followed by screening for regulated protein kinase activity. From these experiments, growth factor stimulated pp90 ribosomal S6 kinases (Rsk) as well as MAP kinases, now known as ERK1 and ERK2, were identified (Erikson and Maller, 1986; Ray and Sturgill, 1987; Jones *et al.*, 1988; Hoshi *et al.*, 1988; Ahn *et al.*, 1990; Boulton *et al.*, 1990), and the enzyme–substrate relationship between ERKs and Rsk was established (Sturgill *et al.*, 1988; Ahn and Krebs, 1990). This was soon followed by biochemical identification of an upstream kinase regulator of ERK, called MAP kinase kinase (MKK), also known as MAP/ERK kinase (MEK) (Ahn *et al.*, 1991; Gomez and Cohen, 1991).

Molecular cloning of ERKs1/2 (Boulton *et al.*, 1990, 1991) and MKK1 (Crews *et al.*, 1992; Kosako *et al.*, 1993) established the homology of these enzymes with yeast FUS3/KSS1 and STE7, respectively, followed by confirmation that STE components were also regulated by direct phosphorylation. Homologous pathways have been identified in all eukaryotic organisms inspected so far. In nearly every case, a MAP kinase homolog regulated by a MKK homolog has been identified, thus the term "MAPK module" was coined to refer to the MAPK/MKK pair (Errede and Levin, 1993). At this point, evidence exists for at least three pathways involving MAPK modules in mammalian systems, five pathways in *cerevisiae*, and three pathways in *Schizosaccaromyces pombe* (Figs. 1 and 2). Functional MAPK modules have also been identified and characterized in frogs, fruit flies, nematodes, slime mold, and plants.

II. MAMMALIAN MAPK PATHWAYS

In mammals, 11 distinct MAPK and 7 MKK genes have been identified to date. Members of the MAPK family include (i) extracellular signal-regulat-

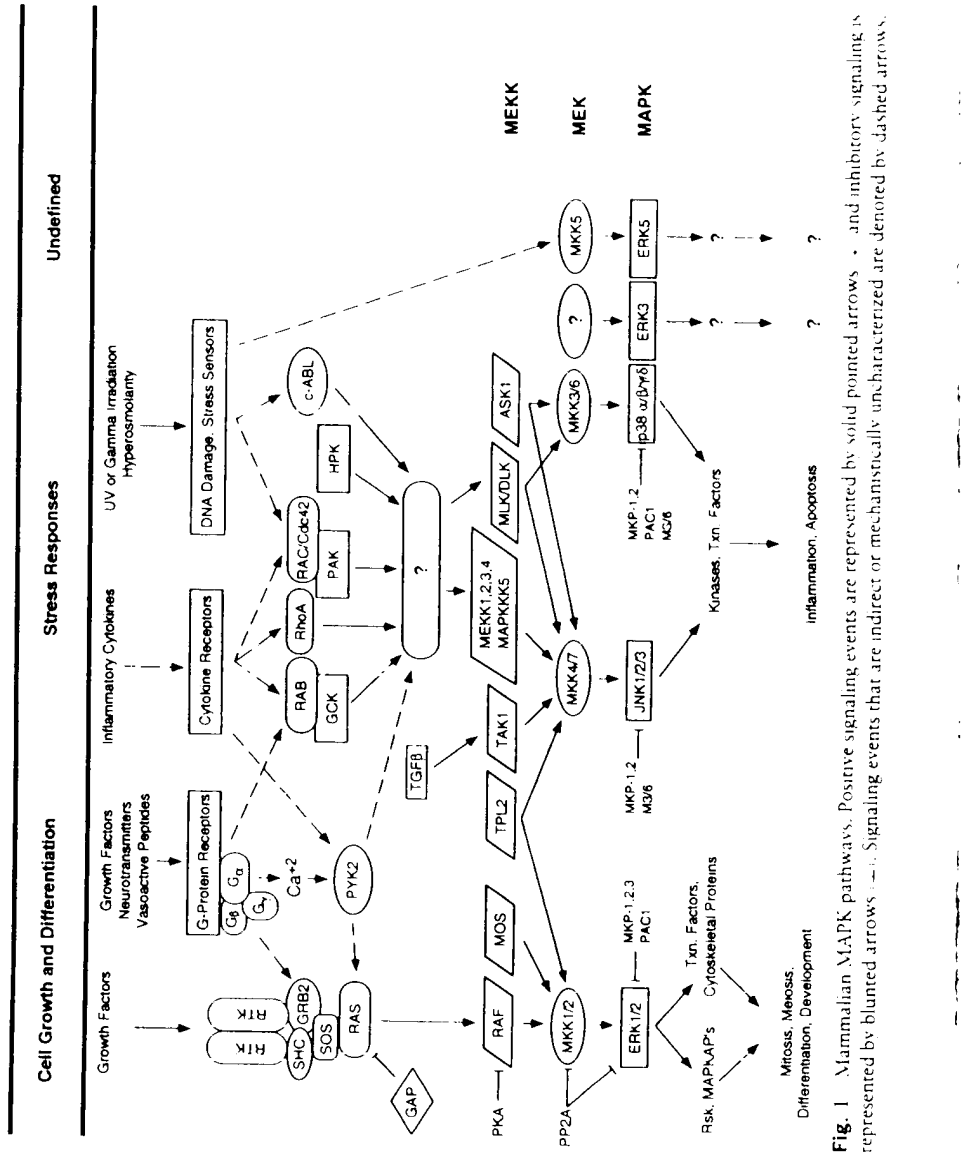


Fig. 1 Mammalian MAPK pathways. Positive signaling events are represented by solid pointed arrows • and inhibitory signaling is represented by blunted arrows —|. Signaling events that are indirect or mechanistically uncharacterized are denoted by dashed arrows.

ed kinases (ERKs) 1 and 2, (ii) NH_2-terminal Jun kinase/stress activated protein kinases (JNK/SAPK) α, β, and γ, and (iii) p38 MAPKs α, β, γ, and δ. These are specifically recognized and phosphorylated by: (i) MKK1 and MKK2 (also known as MEKs 1 and 2), (ii) MKK4 and MKK7, and (iii) MKK3 and MKK6, respectively. Less well characterized is ERK3, for which upstream regulators have not been identified, ERK4, a protein immunocross reactive with antibodies to ERKs 1 and 2, and ERK5 (also known as big molecular weight kinase, BMK), which associates with MKK5. MAPKs and MKKs are closely related to each other by sequence, suggesting that these multicomponent kinase cascades arose through gene duplication of the MAPK module.

MAPKs are activated by MKKs through common mechanisms involving phosphorylation at two regulatory phosphorylation sites with sequence T(P)-X-Y(P) located in the "activation lip" between subdomains 7 and 8 of the conserved kinase core sequence. Thus MKKs fall within a relatively rare class of protein kinases with dual specificity toward Ser/Thr and Tyr residues on exogenous substrates. MKKs are also activated by phosphorylation at Ser/Thr residues within the activation lip. Unlike MAPKs, which are specifically recognized by their corresponding MKKs, each of the MKKs can be phosphorylated and activated by several different MAP kinase kinase kinases (MKKKs), including Raf family members, c-Mos, MEK kinases (MEKKs), and multilineage protein kinases (MLKs). These MKKKs recognize different MKKs, enabling diversity in the activation of MAPK pathways upstream of MKK. As required for switching mechanisms, MAPK cascades transform graded effector signals into cooperative responses. This is a theoretical consequence of pathways involving sequential protein kinases, each regulated by dual phosphorylation, as demonstrated by Huang and Ferrell (1996b).

A. ERK1/2 and MKK1/2 Pathways

ERK1 and ERK2 and their upstream regulators MKK1 and MKK2 are acutely stimulated by growth and differentiation factors, in pathways mediated by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors, or cytokine receptors. At the moment, it is unclear why two forms of each enzyme exist, although conservation of two forms throughout eukaryotic species suggest nonredundant functions. These enzymes are expressed ubiquitously in mammalian cells at micromolar levels (Huang and Ferrell, 1996b), although some variation in expression between different tissues has been noted (Boulton and Cobb, 1991; Moriguchi *et al.*, 1995a). *In vitro*, MKK1 and MKK2 show comparable activity toward ERK1 and ERK2 (Zheng and Guan, 1993a; Dent *et al.*, 1994). A few examples exist where

Fig. 2 MAPK pathways of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Positive signaling is represented by solid pointed arrows (→) and inhibitory events are denoted by solid blunted arrows (—|). The dashed line signifies a protein–protein interaction between STE5 and G_β (STE4). The dashed pointed and blunt arrows in the HOG pathway represent inhibited two-component signaling among SLN1, YPD1, and SSK1 as caused by high extracellular salt concentrations

ERK1 and ERK2 are activated with distinct magnitudes or kinetics following factor stimulation of intact cells (Papkoff *et al.*, 1994; Kalb *et al.*, 1996); however, in most instances, both enzymes are robustly activated on cell stimulation.

1. ERK1 AND ERK2

ERK1 and ERK2 are 44- and 42 kDa enzymes, respectively; in mammals, these have 90% sequence identity (Boulton *et al.*, 1991). In addition to the conserved catalytic core, both enzymes contain C-terminal extensions and a 25 amino acid insert between subdomains 9 and 10. Both enzymes are activated by phosphorylation within their activation lip at Thr_{183}–Glu–Tyr_{185} (numbered as in rat ERK2) in a reaction that is partially ordered with Tyr preceding Thr phosphorylation (Haystead *et al.*, 1992). Both phosphorylation events are required to fully activate wild type ERK1 and ERK2, although 10% activation can be achieved with mutants containing a Thr_{183}-Glu amino acid substitution, presumably due to the substitution of this phosphorylation site with a negatively charged residue (Robbins *et al.*, 1993). Activation by phosphorylation leads to increased V_{max} and decreased K_m for peptide substrate, with little effect on K_{mATP} (Robinson *et al.*, 1996b).

The crystal structure of ERK2 in its inactive, unphosphorylated form was solved by Goldsmith and colleagues to 2.3 Å resolution (Zhang *et al.*, 1994). Similarities within the conserved kinase domain to the structures of other protein kinases were observed, with the inclusion of an additional β sheet structure at the N-terminal lobe (residues 1–20), a novel linker between β4 and β5 (residues 91–96), and an α helical insert between helices G and H in the C-terminal lobe (residues 247–266). Thirty-five C-terminal amino acids wrap in a random coil spanning the C-terminal to N-terminal lobes on the side of the molecule opposite to the catalytic cleft. Recombinant C-terminal residue truncations of ERK2 are poorly expressed (J. Means and N. Ahn, unpublished observations), suggesting that this region may play a role in enzyme stabilization. The two domains of ERK2 are rotated into an open configuration with respect to each other, and the activation lip is distorted with access to the catalytic cleft blocked by the Tyr_{185} phosphorylation site, thus providing an understandable rationale for the low basal activity. In this state, Tyr_{185} is buried whereas Thr_{183} is solvent accessible, suggesting that MKK binding induces a major conformational change in ERK to expose and enable preferential phosphorylation of the Tyr residue. Interestingly, substitution of Tyr_{185} with acidic amino acids led to marked disorder within the activation lip (Zhang, F. *et al.*, 1995). A model to account for these observations postulates induced fit binding of MKK and initial Tyr_{185} phosphorylation on ERK followed by substantial reconfiguration of the lip on Thr_{183} phosphorylation. This stabilizes interactions between phosphorylated residues and catalytic site residues that favor the active conformation (Zhang, F. *et al.*, 1995). Crystallographic determination of the activated ERK2 phosphorylated at Thr_{183} and Tyr_{185} supports this prediction by showing a reorientation of the activation lip, with phosphorylated Thr_{183} bridging residues from both N- and C-terminal lobes and phosphorylated Tyr_{185} forming the substrate binding pocket (Canagarajah *et al.*, 1997).

2. MKK1 AND MKK2

MKK1 and MKK2 are both 44 kDa enzymes, related by 80% sequence identity (Zheng and Guan, 1993a; Wu *et al.*, 1993a). Sequences outside the conserved catalytic core of both enzymes include an additional 60 amino acids at the N terminus and a 40 amino acid insert between subdomains 9 and 10. Activation occurs through phosphorylation at the sequence Ser_{218}(P)–Met–Ala–Asn–Ser_{222}(P) (numbered as in human MKK1) within the activation lip (Zheng and Guan, 1994a; Alessi *et al.*, 1994; Gotoh *et al.*, 1994; Resing *et al.*, 1995). Unlike ERKs, MKKs can be partially activated by phosphorylation at either serine phosphorylation site (Gotoh *et al.*, 1994; Resing *et al.*, 1995). In addition, substitution of these sites by acidic amino acids enhances the basal activity (Zheng and Guan, 1994a; Alessi *et al.*, 1994; Cowley *et al.*, 1994; Pagès *et al.*, 1994; Mansour *et al.*, 1994a). Further enhancement of activity on substituting adjacent nonphosphorylatable residues with acidic amino acids indicates that the activation can be, in part, ascribed to electrostatic effects of the negatively charged residues (Mansour *et al.*, 1996a). The N terminus of MKK outside the conserved kinase subdomain appears to be important in regulating MKK activity, as deletion of residues 44–51 or proline substitution within this region leads to significant activation (Mansour *et al.*, 1994a, 1996a; Bottorff *et al.*, 1995). Alignment of this domain with the A-helix of cAMP-dependent protein kinase suggests potential long range interactions with the activation lip (Herberg *et al.*, 1997). This is supported by the synergistic enhancement of rates between N-terminal deletion and phosphorylation site mutations (Mansour *et al.*, 1996a), as well as by deuterium exchange studies showing that both types of mutations enhance the flexibility within the N-terminal ATP-binding lobe (Resing and Ahn, 1997).

Sequences in MKK1 and MKK2 inserted between subdomains 9 and 10 of the conserved core are proline rich, containing consensus sequences for potential SH3 domain interactions. In MKK1, this domain is important for interactions between MKK and Raf-1 (Jelinek *et al.*, 1994; Papin *et al.*, 1996). Deletion of these residues in a constitutively active MKK background interferes with ERK activation and growth signaling in cells (Catling *et al.*, 1996), suggesting that this domain is involved in substrate recognition *in vivo*, despite the fact that the deletion mutants still phosphorylate ERK *in vitro*. In

MKK1, the insert contains four phosphorylatable residues that are targets for autophosphorylation (Ser_{298}, Tyr_{300}) as well as heterologous phosphorylation by ERK1/2 and cyclin/cdk2 (Thr_{286}, Thr_{292}) or PAK1 (Ser_{298}) (Brunet *et al.*, 1994a; Saito *et al.*, 1994; Mansour *et al.*, 1994b; Rossomando *et al.*, 1994; Resing *et al.*, 1995; Frost *et al.*, 1997). In MKK2, phosphorylatable residues are only found at Thr_{292} and Ser_{298}. Phosphorylation of these sites could potentially mediate MKK/Raf-1 interactions (Jelinek *et al.*, 1994), feedback regulation of MKK activity by ERK (Brunet *et al.*, 1994a), or cross regulation of MKK1 with PAK-dependent signaling pathways (Frost *et al.*, 1997).

In vitro, MKK1 and MKK2 are targets for phosphorylation and activation by at least three protein kinase families. Raf-1 and B-Raf phosphorylate MKK1 and MKK2, whereas A-Raf is a weak activator of MKK1/2 in intact cells and shows greater selectivity for MKK1 *in vitro* (Wu, X. *et al.*, 1996; Pritchard *et al.*, 1995). MKK1 and MKK2 are also activated by the germ cell specific protein kinase, c-Mos, which controls meiotic cell division (Posada *et al.*, 1993; Nebreda *et al.*, 1993). Because MEKK and several isoforms are potent regulators of stress-activated protein kinase pathways, the discussion of MEKKs will be deferred to the next section. However, MEKKs and Tpl-2 phosphorylate MKK1 *in vitro* and under some conditions of expression *in vivo* (Yan and Templeton, 1994; Xu *et al.*, 1995; Blank *et al.*, 1996; Salmerón *et al.*, 1996). These enzymes phosphorylate both of the serine residues within the activation loop, although Mos preferentially phosphorylates Ser_{222}, MEKK1 and STE11 prefer Ser_{218}, and Raf-1 phosphorylates both sites equally well (Yan and Templeton, 1994; Alessi *et al.*, 1994; Gotoh *et al.*, 1994; Resing *et al.*, 1995). *In vitro*, this activation is followed by intramolecular autophosphorylation at Thr_{23}, Ser_{298}, and Tyr_{300} (Resing *et al.*, 1995).

3. Raf

Raf can be regulated by several mechanisms, the combination of which leads to full activation by growth regulatory signals. In addition to the C-terminal catalytic domain (amino acids 335–627), Raf-1, A-Raf, and B-Raf contain N-terminal regulatory domains within a conserved region 1 (CR1, amino acids 61–194 numbered as in Raf-1), which include a Ras-binding domain (RBD, amino acids 51–131) and a cysteine-rich domain containing a zinc finger motif (amino acids 139–184). A conserved region 2 (CR2, amino acids 254–269) and the very C terminus are important for binding the regulatory 14-3-3 protein.

A basic model for Raf-1 regulation involves membrane recruitment, enabling its interactions with upstream regulators. Raf-1 recruitment is mediated by Ras-GTP (Marais *et al.*, 1995), involving interactions between the Ras-binding and Cys-rich domains of Raf, respectively, with Switch I (amino acids 30–38) and Switch II (amino acids 60–76) effector regions of Ras GTP (Moodie *et al.*, 1993; Wojtek *et al.*, 1993; Hu, C. *et al.*, 1995; Brtva *et al.*, 1995; Drugan *et al.*, 1996). The GTP/GDP exchange of p21Ras is catalyzed by Ras guanine nucleotide exchange factors, including mammalian son of-sevenless (Sos). Growth factor stimulation of GTP/GDP exchange entails recruitment of Grb2-Sos to membrane-bound Ras via direct interactions between receptor and Grb2 or indirect interactions through adaptor proteins such as Shc or IRS-1 (reviewed by Pawson, 1995). Disruption of either interaction leads to the suppression of transformation by v-ras, thus both interactions are important for Ras signaling (Fridman *et al.*, 1994; Brtva *et al.*, 1995). The importance of membrane recruitment was illustrated by the observation that membrane targeting of Raf-1 by C terminal farnesylation or N-terminal myristylation transforms cells without participation of Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). A cocrystal structure of Raf-RBD and Rap complexed with a nonhydrolyzable GTP analog reveals close contacts between residues in the Raf RBD and residues conserved between Ras and Rap (Nassar *et al.*, 1995), suggesting how analogous interactions between Ras and Raf might be structured.

Phosphorylation of Raf-1 appears to be a key regulatory mechanism. After purification from Sf9 cell expression, Raf-1 is phosphorylated at Ser_{43}, Ser_{259}, Ser_{621}, Tyr_{340} and Tyr_{341}, and autophosphorylated at Thr_{268} (Fabian *et al.*, 1993a; Morrison *et al.*, 1993). Raf-1 activation appears to require phosphorylation at one or both tyrosine residues because mutation of these sites blocks activation by Ras and because protein tyrosine phosphatases inactivate Raf-1 (Dent *et al.*, 1995; Jelinek *et al.*, 1996). Likely candidates for regulatory tyrosine kinases are Src and Fyn, based on studies demonstrating Raf-1 activation on coexpression with Src in a manner dependent on $Tyr_{340/341}$ (Fabian *et al.*, 1993; Cleghon *et al.*, 1994; Marais *et al.*, 1995). Raf-1 activation by Src requires a functional RBD, suggesting that membrane recruitment is required for tyrosine phosphorylation (Marais *et al.*, 1995). Ras–Raf interactions are not as important in Sf9 coexpression systems, presumably due to high expression levels (Fabian *et al.*, 1994). Other candidates are the JAK tyrosine kinases, which appear to regulate Raf-1 in systems involving signaling through receptors for cytokines such as erythropoetin, interferon-γ, and growth hormone (Miura *et al.*, 1994; Winston and Hunter, 1995; Xia *et al.*, 1996). JAK2 directly phosphorylates Raf-1 *in vitro*, although $Tyr_{340/341}$ account for only 75% of the incorporated label from ^{32}P-ATP (Xia *et al.*, 1996). Involvement of serine/threonine kinases is indicated by reports of Raf-1 activation following its direct phosphorylation by protein kinase C (PKC) at Ser_{499} (Sozeri *et al.*, 1992; Kolch *et al.*, 1993; Carroll and May, 1994) or by a ceramide-activated protein kinase at Thr_{269} (Yao *et al.*, 1995). Inhibition of Raf-1 on phosphorylation by cAMP-dependent protein kinase at Ser_{43} or Ser_{621} has also been reported (Wu *et al.*, 1993b;

Mischak *et al.*, 1996); phosphorylation at Ser_{621} may be required for Raf activation through its interaction with 14-3-3 (Muslin *et al.*, 1996). ERK also phosphorylates Raf-1, with no clear-cut effect *in vitro*, although it may suppress Raf-1 activity *in vivo* (Anderson *et al.*, 1991; Ueki *et al.*, 1994). In support of this, cellular stimulation of ERK leads to retardation of Raf gel mobility, which correlates with $Ser_{621/624}$ phosphorylation and lowered Raf activity (Ueki *et al.*, 1994; Ferrier *et al.*, 1997).

Raf-1 heterocomplex formation has been proposed to be an important aspect of regulation. Physiologically, Raf-1 oligomerization may be accomplished through its interaction with 14-3-3β, η, ζ and θ (Fantl *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994; Aitken *et al.*, 1995; Papin *et al.*, 1996), members of a large conserved acidic protein family that interact with many targets, including Raf-1, B-Raf, Bcr, polyomavirus middle T antigen, cdc25 phosphatases, phosphatidylinositol 3′ kinase, Cbl, and protein kinase C-θ (reviewed by Aitken 1995; Meller *et al.*, 1996). Residues within the C terminus of 14-3-3 interact with CR2 and the C terminus of Raf-1, recognizing phosphoserine at Ser_{259} and Ser_{621} (Luo *et al.*, 1995; Muslin *et al.*, 1996). The importance of this interaction on Raf-1 regulation is indicated by several studies demonstrating activation of Raf-1 by 14-3-3 on coexpression in cells or in cell-free extracts (Freed *et al.*, 1994; Fantl *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994; Li *et al.*, 1995). However, activation has not been observed in all cases (Michaud *et al.*, 1995; Suen *et al.*, 1995), and, in fact, 14-3-3 binding may stabilize an inactive form of Raf, based on Raf activation by disruption of 14-3-3 interactions. The ability of 14-3-3 to dimerize raises the possibility that 14-3-3 proteins may mediate the heterocomplex formation of Raf-1 with other signaling molecules. For example, Raf-1 association with Bcr has been demonstrated, mediated by their mutual interaction with 14-3-3 (Braselmann and McCormick, 1995).

A 14-3-3ζ mutant that is unable to dimerize binds efficiently to Raf-1, but favors the inactive form of the kinase, suggesting that dimerization may be needed for Raf activation (Luo *et al.*, 1995). Consistent with this, activation of basal Raf-1 and MKK1/2 activity was demonstrated on homodimerization of FKBP-Raf-1/cyclophilin or bacterial DNA gyrase-Raf-1 chimeras in response to bifunctional ligands FK1012A or coumermycin, respectively (Luo *et al.*, 1996; Farrar *et al.*, 1996). *In vitro*, Raf-1 elutes by gel filtration within protein complexes greater than 300 kDa containing several proteins with chaperone function, such as hsp90, Cdc37, and hsp56 (Wartmann and Davis, 1994; Stancato *et al.*, 1994). Formation of hsp90 complexes appears to be important for Raf-1 signaling because the hsp90 ligand, geldanamycin, blocks growth factor signaling through the disruption of Raf-1/MKK complexes (Schulte *et al.*, 1996). However, no evidence exists for growth factor regulation of Raf-1:hsp90 association or dissociation. Most likely, hsp90 interacts with Raf during folding, although hsp90 and 14-3-3 binding to Raf

also protect against Raf-1 dephosphorylation and inactivation by PTP1B (Jelinek *et al.*, 1996).

Two additional interacting proteins may be important for Raf-1 activation. The kinase suppressor of Ras (KSR), a protein kinase closely related to Raf, was identified as a positive modifier of Ras signaling in *Drosophila melanogaster* and *Caenorhabditis elegans* (Kornfeld *et al.*, 1995a; Sundaram and Han 1995; Therrien *et al.*, 1995). Although direct regulation of Raf-1 by KSR has not been observed, biochemical experiments show association of human KSR with Raf, MKK1, and ERK and enhancement of signaling through the MKK/ERK pathway on coexpression with v-Ras in mammalian cells or *Xenopus* oocytes (Therrien *et al.*, 1996). Direct stimulation of Raf-1 by BAG-1, a protein that binds to and enhances the antiapoptotic effects of Bcl-2, has been demonstrated *in vitro* (H. H. Wang *et al.*, 1996). Thus, BAG-1 may bind to and recruit Raf to Bcl-2. Involvement of Raf-1 in Bcl-2 regulation of apoptosis is suggested by biochemical interactions between Raf-1 and Bcl-2 and the observation that Bcl-2 and the proapoptotic Bcl2 homolog, BAD, are both substrates for phosphorylation by Raf-1 (Wang *et al.*, 1994; Blagosklonny *et al.*, 1996; Zha *et al.*, 1996).

Other potential membrane activators of Raf-1 include lipids and lipid metabolites. *In vitro*, Raf-1 can be activated by direct phorbol ester binding, mediated through its zinc finger domains, which are homologous to those on several PKC isoforms (Luo *et al.*, 1997). Raf-1 also binds to phosphatidylserine, phosphatidic acid, and ceramide (Ghosh *et al.*, 1996; Huwiler *et al.*, 1996). Binding of ceramide enhances Raf-1 activity *in vitro* and *in vivo*, and inhibition of phosphatidic acid synthesis in cells blocks Raf-1 membrane translocation. Finally, Raf-1 activation has been shown under certain conditions to be retarded by the inhibition of phosphatidylinositol 3′ kinase, suggesting a possible role for lipid products of this enzyme in regulating Raf-1 activity (Cross *et al.*, 1994; J. Huang *et al.*, 1995).

In contrast to Raf-1, which is ubiquitous, other forms of Raf show more restricted tissue expression. A-Raf is mainly expressed in steroid responsive (e.g., urogenital) tissues (Winer and Wolgemuth, 1995; Lee *et al.*, 1996). Several isoforms of B-Raf exist, many of which are selectively expressed in neuronal tissue (Barnier *et al.*, 1996). Comparison of B-Raf to Raf-1 reveals similar but nonidentical mechanisms of regulation. Like Raf-1, B-Raf interacts with Ras-GTP and forms large molecular weight complexes that include hsp90 and 14-3-3 (Moodie *et al.*, 1994; Jaiswal *et al.*, 1996; Papin *et al.*, 1996); however, unlike Raf-1, B-Raf complexed with 14-3-3 proteins retains its activity (Yamamori *et al.*, 1995). B-Raf has a higher basal activity than Raf-1, ascribed to negatively charged Asp residues at positions equivalent to Tyr_{340} and Tyr_{341} in Raf-1, and it is not as highly phosphorylated on tyrosine in response to factor stimulation, although it is still phosphorylated on Ser/Thr (Stephens *et al.*, 1992; Jelinek *et al.*, 1996). Moreover, B-Raf is stim-

ulated by interaction with Rap1-GTP (Ohtsuka *et al.*, 1996; Vossler *et al.*, 1997), whereas Rap1B-GTP binds but does not lead to Raf-1 activation (Zhang *et al.*, 1993). Dependence on phospholipid activators also differ; thus, phosphatidylserine stimulates B-Raf activity in a Ras-dependent manner, whereas phosphatidylinositol, phosphotidylserine, and phosphatidylethanolamine all inhibit Rap1B-stimulated B-Raf activation (Kuroda *et al.*, 1996).

4 Mos

The serine/threonine protein kinase c-Mos regulates meiosis in vertebrate oocytes (Singh and Arlinghaus 1992; Yew *et al.*, 1993). Its specific expression in germ cells is regulated by somatic cell repressor DNA-binding elements within the Mos promoter (Xu and Cooper, 1995), although somatic cell expression has also been reported (Leibovitch *et al.*, 1993; Gao *et al.*, 1996). *Xenopus laevis* oocytes stimulated to undergo meiotic cell division upregulate Mos protein levels by cytoplasmic 3′ polyadenylation and stabilization of message (Sheets *et al.*, 1994, 1995; Gebauer *et al.*, 1994; Stebbins-Boaz *et al.*, 1996). Meiotic cell division, which can be induced by injection of Mos mRNA or protein into oocytes, requires activation of MKK and ERK; these are maximally active at metaphase I and II (Kosako *et al.*, 1994a; Gotoh *et al.*, 1995a; Roy *et al.*, 1996). *In vitro*, Mos phosphorylates MKK1 at regulatory Ser_{218} and Ser_{222} phosphorylation sites (Posada *et al.*, 1993; Nebreda *et al.*, 1993; Resing *et al.*, 1995), and maturation through both meiosis I and II can be mimicked by the injection of constitutively active mutant MKK1 or thiophosphorylated ERK1 (Haccard *et al.*, 1995; Gotoh *et al.*, 1995a), thus MKK is a key target for Mos in this process. Regulation of Mos- or progesterone-dependent meiosis also involves Raf-1 activation, as indicated by the interference of germinal vesicle breakdown on the expression of inactive dominant negative Raf mutants (Muslin *et al.*, 1993; Fabian *et al.*, 1993); however, the ability of both Raf-1 and Mos to directly activate MKK suggests convergence of Raf and Mos signaling rather than sequential activation.

Maturation of oocytes into meiosis II is inhibited by cycloheximide. This is due in part to a requirement for further synthesis of Mos protein, in a secondary induction that appears to require ERK activity (Yew *et al.*, 1992; Gotoh *et al.*, 1995a; Roy *et al.*, 1996; Matten *et al.*, 1996). Phosphorylation of Mos at Ser_3 enhances Mos protein levels (Nishizawa *et al.*, 1992) and may lead to stable MKK interactions (Chen and Cooper, 1995). Ser_3 is phosphorylated by ERK *in vitro*, suggesting that in addition to message stabilization, Mos is regulated by positive feedback involving direct phosphorylation by ERK (Matten *et al.*, 1996).

Importantly, Mos functions as a cytostatic factor, elevating MPF activity and thus maintaining metaphase II arrest prior to fertilization. Following fertilization, calpain-mediated degradation of Mos is followed by a rapid decay of ERK activity, releasing cell cycle arrest. That ERK is a key component of cell cycle arrest is indicated by the cytostatic arrest of two cell embryos on microinjection of constitutively active MKK or thiophosphorylated ERK (Haccard *et al.*, 1993; Kosako *et al.*, 1994b). Targeted gene disruption of Mos in mice yields mouse oocytes that are unable to arrest in metaphase II and undergo parthenogenetic activation (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). Based on studies examining the effects of Mos on meiosis, ERK is implicated in metaphase arrest, meiotic spindle formation, and polar body degradation, and in the prevention of pronuclear envelope formation until fertilization (Verlhac *et al.*, 1993; Moos *et al.*, 1996; Choi *et al.*, 1996 a,b).

MPF activation by Mos likely involves protection of cyclin B from proteolysis. Although some studies have shown suppression of cdc2/cyclinB activation by dominant-interfering ERK mutants, the results have been mixed and a direct link with the MAPK pathway has not been established (Nebreda and Hunt, 1993; Pomerance *et al.*, 1996; Huang and Ferrell 1996a). However, egg extracts induced to undergo spindle checkpoint arrest show high ERK activity and cyclin B levels. Arrest is released on inactivation of ERK by MAP kinase phosphatase-1, suggesting that ERK may also affect the mechanisms involved in cyclin degradation (Minshull *et al.*, 1994).

5 SIGNALING THROUGH INTRACELLULAR CALCIUM

MKK/ERK can be stimulated by elevation of intracellular calcium with calcium ionophores, thapsigargin, elevated extracellular calcium, or membrane depolarization (Chao *et al.*, 1994; Rosen *et al.*, 1994; S. Huang *et al.*, 1995; Kurino *et al.*, 1995; Bogoyevitch *et al.*, 1996). In many cases, activation of Raf-1 and/or Ras in addition to MKK/ERK can be demonstrated, suggesting regulation of targets upstream of Ras. One candidate is Pyk2, a member of the focal adhesion kinase (FAK) family of nonreceptor protein tyrosine kinases, which is expressed in neuronal cells and tyrosine phosphorylated in response to calcium influx and membrane depolarization (Lev *et al.*, 1995; Dikic *et al.*, 1996). Forced expression of Pyk2 activates ERK, and dominant negative mutant Pyk2 blocks ERK activation by carbachol. Pyk2 also associates with Grb2 and Sos in intact cells following membrane depolarization or carbachol treatment of PC12 cells, suggesting a mechanism for its activation of Ras.

An alternative (but potentially related) means of activating MKK/ERK by calcium involves Ca^{2+}-calmodulin (CaM)-dependent protein kinases. Overexpression of Ca^{2+}-CaM kinase IV in combination with its activator, CaM kinase kinase, led to a two- to fivefold activation of several MAPKs in PC12 cells, including JNK/SAPK, p38, and ERK2 (Enslen *et al.*, 1996). An involvement of Ca^{2+}-CaM kinase II in the regulation of MKK/ERK is also sug-

gested by the inhibition of norepinephrine activation of ERK by the Ca^{2+}-CaM kinase II inhibitor KN-93 in aortic smooth muscle cells (Muthalif *et al.*, 1996). However, the involvement of calmodulin-regulated kinases in activating upstream regulators such as Raf-1 or Ras has not been established.

6. SIGNALING THROUGH HETEROTRIMERIC G PROTEINS

Activation of MKK/ERK by heterotrimeric $G_{\alpha i}$, $G_{\alpha o}$, or $G_{\alpha q}$ protein-coupled receptors occurs in response to many agonists (e.g., lysophosphatidic acid, carbachol, endothelin, prostaglandin F_2, bombesin, and thrombin). Overexpression of constitutively active GTPase-deficient $G_{\alpha i}$, $G_{\alpha o}$, or $G_{\alpha q}$ mutants in various cell lines also leads to MKK/ERK activation (Gupta *et al.*, 1992; Faure *et al.*, 1994; Pace *et al.*, 1995). $G_{\beta\gamma}$ subunits appear to be involved in the mechanism of $G_{\alpha i}$-coupled ERK regulation because activation can be suppressed by peptides corresponding to the C-terminal βγ-binding domain of the β-adrenergic receptor kinase (Koch *et al.*, 1994) and because ERK is activated on expression of $G_{\beta\gamma}$ subunits (Faure *et al.*, 1994; Crespo *et al.*, 1994; Ito *et al.*, 1995; Hawes *et al.*, 1995). $G_{\beta\gamma}$ subunits appear to activate Ras, Raf, MKK, and ERK through a PKC-independent mechanism that involves tyrosine phosphorylation of Shc and signaling to Ras through Shc Grb2-Sos interactions (Van Biesen *et al.*, 1995; Hawes *et al.*, 1995). The tyrosine kinases Src and Syk are implicated in this process (Luttrell *et al.*, 1996; Wan *et al.*, 1996). In addition, the sensitivity of this pathway to wortmannin suggests a role of phosphatidylinositol 3′ kinase downstream of $G_{\beta\gamma}$ and upstream of Ras (Hawes *et al.*, 1996). G-protein-coupled α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) activation of ERK resulted in association of $G_{\beta\gamma}$ with a complex containing Ras, Raf, MEK, and ERK (Wang and Durkin, 1995), indicating that heterocomplex formation may occur between kinases and heterotrimeric G proteins.

In contrast to $G_{\alpha i}$, stimulation of MKK/ERK through $G_{\alpha q}$- or $G_{\alpha o}$-coupled receptors is independent of $G_{\beta\gamma}$ or Ras and instead activates Raf/MKK/ERK through a PKC-dependent mechanism (Van Biesen *et al.*, 1996; Hawes *et al.*, 1996). This may be a cell type-specific result because signaling through $G_{\alpha q}$ in cardiac myocytes or CCL39 fibroblasts led to Ras/Raf activation through Shc-Grb2-Sos interactions in a PKC-independent manner (Y. Chen *et al.*, 1996b; Sadoshima and Izumo, 1996). Candidate tyrosine kinase mediators of ERK activation through $G_{\alpha q}$ coupled signaling are Lyn or Syk (Wan *et al.*, 1996). Pyk2 appears to play an important role in heterotrimeric G protein signaling as well because ERK stimulation by agonists of $G_{\alpha i}$- or $G_{\alpha q}$-linked pathways was suppressed by the dominant negative mutant Pyk2 in a process dependent on Src activation (Dikic *et al.*, 1996). Under these conditions, Grb2 and Sos were required for ERK activation, indicating that signaling is Ras dependent.

$G_{\alpha s}$-mediated signaling through cAMP-dependent protein kinase suppresses MKK/ERK activation in many systems (Wu *et al.*, 1993b; Sevetson *et al.*, 1993; Graves *et al.*, 1993; Cook and McCormick, 1993; Hordijk *et al.*, 1994), providing an explanation for the commonly observed antagonistic effects of cAMP on cell growth. One target for cAMP-dependent protein kinase inhibition is Raf-1, which is directly phosphorylated at Ser_{43} and Ser_{621} *in vitro* (Wu *et al.*, 1993b; Mischak *et al.*, 1996). However, inhibition of ERK signaling is not observed in all cells and cAMP modulates the kinetics but not the magnitude of ERK activation under some conditions (McKenzie and Pouysségur, 1996). In PC12 cells, agonists of cAMP signaling actually stimulate MKK/ERK (Frodin *et al.*, 1994; Young *et al.*, 1994; Vossler *et al.*, 1997). This appears to result from the predominant role of B-Raf in regulating the MKK/ERK pathway in PC12 cells in that cAMP activates guanine nucleotide exchange of Rap1, which in turn activates B-Raf in a Ras-independent manner (Vossler *et al.*, 1997).

7. SIGNALING THROUGH PROTEIN KINASE C

A long-standing problem that is still unresolved is the mechanism by which PKC regulates MKK and ERK. Phorbol esters activate ERK in nearly every cell, and many reports exist of agonist stimulation of ERK that is at least partially blocked on PKC inhibition by extended phorbol ester treatment, low molecular weight inhibitors, or dominant negative PKC mutants (Hoshi *et al.*, 1989; Ueda *et al.*, 1996; Hundle *et al.*, 1995; Berra *et al.*, 1995). In lymphocytes, phorbol 12-myristate 13-acetate (PMA) leads to Ras GTP elevation (Downward *et al.*, 1990), and activation of ERK by PKC in broken cell extracts has been shown to require Ras (VanRenterghem *et al.*, 1994). Therefore, one effect of PMA is to probably regulate Raf through the activation of Ras. The Pyk2 tyrosine kinase is a candidate for a Ras activator because it is also stimulated by phorbol ester (Lev *et al.*, 1995). This may explain why PMA-dependent ERK activation is sensitive to tyrosine kinase inhibitors (Seger *et al.*, 1995). However, Ras-independent activation of Raf-1 has also been observed (Ueda *et al.*, 1996). An alternative mechanism to account for this involves direct phosphorylation of Raf by PKC-γ, resulting in Raf-1 activation *in vitro*, as mentioned earlier (Sozeri *et al.*, 1992; Kolch *et al.*, 1993; Carroll and May, 1994). Interestingly, a Raf-1 chimera substituting the zinc finger domain with the analogous region from PKC is strongly responsive to phorbol ester in a manner that is unaffected by phorbol ester downregulation of PKC, suggesting that the zinc finger in Raf may be involved in the response to PMA (Luo *et al.*, 1997). Although a direct comparison of the effects of individual PKC isoforms on MKK/ERK activation is still lacking, various reports have documented the involvement of PKC α, βI, γ, δ, ϵ, and ι in MKK/ERK activation (Ueda *et al.*, 1996; Yamaguchi *et al.*, 1995a; Hundle *et al.*, 1995; Berra *et al.*, 1995; Young *et al.*, 1996).

8. SIGNALING THROUGH JAK/STAT PATHWAYS

Cytokine signaling pathways lead to interactions between receptors and members of the Janus family of tyrosine kinases (JAKs1, 2, and 3, TYK2), resulting in transphosphorylation and activation of kinase activity. STAT transcription factors are recruited to the receptor complex via SH2 domain interactions where they become tyrosine phosphorylated and dimerize through intermolecular interactions between N-terminal SH2 domains and C-terminal phosphotyrosine motifs (reviewed by Ihle, 1996). The resulting hetero- or homodimers translocate to nuclei where they mediate transcription by interaction with DNA response elements within cytokine regulated enhancers, such as the interferon γ site (GAS) response element. The cytokine regulation of MKK/ERK in some cases may be explained by Janus kinase activation because growth hormone stimulation of ERK2 is blocked by dominant interfering mutants of JAK2 (Winston and Hunter, 1996). Expression of dominant negative mutant ERK2 blocks interferon β-induced transcription, and treatment of cells with the MKK1 inhibitor, PD98059, blocks IL-6 induced transcription, indicating a role for ERK in cytokine signaling through STAT1 or STAT3 (David *et al.*, 1995; Bhat *et al.*, 1996). A reasonable model would involve JAK dependent activation of Ras through a Shc/Grb2 coupled pathway, as documented in several cases (VanderKuur *et al.*, 1995; He *et al.*, 1995; Chauhan *et al.*, 1995). However, not all cytokine receptor pathways utilize JAK for activating ERKs (Miura *et al.*, 1994), indicating alternative mechanisms for carrying out these processes.

Cross regulation between JAK and ERK pathways also involves STAT regulation by ERK phosphorylation. Serine phosphorylation of STAT1 and STAT3 is necessary for full transcriptional activity, most likely stabilizing STAT dimers and STAT–DNA complex formation (Wen *et al.*, 1995; X. Zhang *et al.*, 1995). Phosphorylation of STAT1 or STAT3 at Ser_{727} is important for this effect. These sites lie within the consensus sequence for ERK phosphorylation (Pro-X-Ser(P)-Pro), thus allowing positive feedback of STAT signaling by MKK/ERK.

9. SIGNALING THROUGH PHOSPHATIDYLINOSITOL 3′ KINASE

Evidence for an involvement of phosphatidylinositol 3′ kinase (PI3′K) on MKK/ERK activation is based primarily on the suppression of signaling by pharmacological inhibitors or dominant negative mutants of PI3′K. Although PI3′K signaling is likely to be more important in stress-activated kinase pathways, the PI3′K inhibitors wortmannin or Ly294002 (Powis *et al.*, 1994; Vlahos *et al.*, 1994) inhibit ERK activation in response to insulin, serum, vasopressin, platelet-activating factor, growth hormone, $G_{\beta\gamma}$ subunit expression, and T-cell receptor activation (Cross *et al.*, 1994; Welsh *et al.*, 1994; Ferby *et al.*, 1994; Nishioka *et al.*, 1995; Urich *et al.*, 1995; Kilgour *et al.*, 1996; Von Willebrand *et al.*, 1996). Supporting evidence shows inhibition of ERK following the expression of dominant negative mutant PI3′K and ERK activation by constitutively active mutant PI3′K (Hu, Q. *et al.*, 1995b; Von Willebrand *et al.*, 1996). However, various studies have yielded conflicting results. In some cases, wortmannin or Ly294002 blocks ERK activation at doses uncorrelated with their inhibition of PI3′K, suggesting nonspecific mechanisms of action (Frevert and Kahn, 1997). Regulation of ERK by PI3′K might be mediated through Ras, which binds directly to the p110 catalytic subunit, resulting in elevation of PI3′K activity (Rodriguez-Viciana *et al.*, 1996).

B. Stress-Activated Protein Kinase Pathways

1. STRESS-ACTIVATED MAPKs: JNK/SAPK AND p38 MAPK

NH_2-terminal cJun kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAP kinase are implicated in responses to cellular stress, inflammation, and apoptosis. Members of both families are ubiquitously expressed and are activated in response to lipopolysaccharides and proinflammatory cytokines interleukin-1 or tumor necrosis factor (TNF-α), ionizing or ultraviolet (UV) radiation, inhibitors of translation such as cycloheximide or anisomycin, cancer chemotherapeutics, tumor promoters, heat shock or hyperosmotic stress (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Han *et al.*, 1994; Raingeaud *et al.*, 1995; Y. Chen *et al.*, 1996a; Kuroki *et al.*, 1996; Osborn and Chambers, 1996). These are distinct from cell growth and transformation responses controlled by MKK/ERK, although the stress-activated kinases can be activated by growth stimuli under some conditions (Fig. 1).

JNK/SAPK was first identified through purification as a cycloheximide-activated MAP2 kinase (Kyriakis and Avruch, 1990) as well as a protein kinase that copurified with and phosphorylated c-Jun (Adler *et al.*, 1992; Hibi *et al.*, 1993). Subsequent cloning efforts revealed three genes encoding JNK/SAPK isoforms: JNK1, JNK2, and JNK3 (corresponding to SAPKα, β, and γ) (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). Alternative mRNA splicing at the C terminus results in further diversification of the JNK/SAPKs into as many as 10 isotypes, including four forms of JNK1, four forms of JNK2, and two forms of JNK3 with molecular masses ranging from 45 to 57 kDa (Gupta *et al.*, 1996).

p38 MAPK was first purified as a protein phosphorylated in response to lipopolysaccaride (Han *et al.*, 1994) and as a "reactivating kinase" able to phosphorylate and activate phosphatase-treated MAPKAP kinase-2 (Rouse *et al.*, 1994). Both studies led to cloning of a protein kinase sharing 52% amino acid identity with the *S. cerevisiae* HOG1 protein kinase that responds

to osmotic stress (Brewster *et al.*, 1993; Han *et al.*, 1994). The functional similarity of JNK/SAPK and p38 MAPK was established by complementation of yeast hog1Δ strains (Han *et al.*, 1994; Galcheva-Gargova *et al.*, 1994). Four forms of p38 MAPK (α, β, γ, and δ, also known respectively as RK/SAPK2a, SAPK2b, ERK6/SAPK3, and SAPK4), have been identified (Jiang *et al.*, 1996; Z. Li *et al.*, 1996; Goedert *et al.*, 1997). p38 MAPK α and β are inhibited by novel cytokine suppressive anti-inflammatory drugs (CSAIDs) (Lee *et al.*, 1994), are sometimes referred to as CSAID-binding proteins (CSBP1 and CSBP2). An alternatively spliced variant of p38 MAPK, called Mxi2, is a C-terminal truncation of p38 MAPKα, discovered by its interaction with Myc and its interacting partner, Max (Zervos *et al.*, 1995).

The overall sequence identity among JNK/SAPK, p38 MAPK, and ERKs is 40–45%. All three enzymes share a common mechanism for activation through phosphorylation of a Thr–X–Tyr motif found in the activation lip (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Gupta *et al.*, 1996). JNK/SAPK is activated by the phosphorylation of Thr–Pro–Tyr, whereas p38 MAPK is phosphorylated at a Thr–Gly–Tyr motif. The length of the activation lip differs among ERK, JNK/SAPK, and p38 MAPK, which contain 25, 21, and 19 amino acid residues, respectively, which are located between the Asp–Phe–Gly and Ala–Pro–Glu conserved sequences in subdomains 7 and 8. The sequences in the N-terminal half of the activation lip also differ among the three MAPKs. However, these differences do not account for the specific recognition by upstream protein kinases because substitution of the ERK2 activation lip by p38 MAPK or JNK/SAPK sequences does not affect ERK2 phosphorylation by MKK1 (Robinson *et al.*, 1996a). Sequences immediately C-terminal to the P+1 recognition site are similar in all three MAPKs, consistent with their common recognition of Ser/Thr–Pro motifs. Crystallographic studies of the inactive form of p38 MAPKα show similarities in the overall domain structure, but significant differences in the folding of the activation lip compared to that of inactive ERK2 (Wilson *et al.*, 1996; Z. Wang *et al.*, 1997). Presumably, conformational changes induced by phosphorylation of p38 MAPK lead to an activation lip structure similar to that of active ERK2.

2. STRESS-ACTIVATED MAP KINASE KINASES 3, 4, 6, AND 7

Polymerase chain reaction (PCR) strategies were used to identify the upstream activators of JNK/SAPK and p38 MAPK from mammalian libraries, using yeast PBS2 (Brewster *et al.*, 1993), Xenopus XMEK2 (Yashar *et al.*, 1993), or *Drosophila hemipterous* (Glise *et al.*, 1995) as templates (Sánchez *et al.*, 1994; Dérijard *et al.*, 1995; Raingeaud *et al.*, 1996). To date, four distinct MKKs have been identified (Fig. 1). MKK3 selectively phosphorylates p38 MAPK (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1996). MKK6 is closely

related to MKK3 (80% sequence identity) and also phosphorylates p38 MAPK, although its basal activity is approximately 300-fold higher than that of MKK3 (Raingeaud *et al.*, 1996; Han *et al.*, 1996; Moriguchi *et al.*, 1996b; Stein *et al.*, 1996). Both MKK3 and MKK6 have been found in two splice variant forms (Han *et al.*, 1996; Moriguchi *et al.*, 1996a). MKK4 and MKK7 (also called JNKK1/SEK1 and JNKK2, respectively) are regulators of JNK/SAPK, although MKK4 also phosphorylates p38 MAPK *in vitro* (Sánchez *et al.*, 1994; Dérijard *et al.*, 1995; Lin *et al.*, 1995; Tournier *et al.*, 1997). MKKs 3, 4, 6, and 7 range from 35 to 45 kDa and lack the proline rich insertion between subdomains 9 and 10 that is found in MKK1 and MKK2, although MKK4 retains a consensus site for ERK phosphorylation at its N terminus and is a substrate for phosphorylation by ERK (Dérijard *et al.*, 1995).

MKKs 3, 4, 6, and 7 are responsive to inflammatory cytokines, ultraviolet or γ irradiation, translation inhibitors, and osmotic stress, as expected from the behavior of JNK/SAPK and p38 MAPKs. In some cells, MKK4 may be preferentially activated by translation inhibitors, whereas MKK3 and MKK6 may be preferentially activated by hyperosmotic shock, although this appears to vary substantially according to cell type (Moriguchi *et al.*, 1995b, 1996a; Cuenda *et al.*, 1996; Meier *et al.*, 1996; Zanke *et al.*, 1996a). The activation lip of these enzymes all contain serine residues at the position homologous to Ser_{218} in hMKK1 and threonines at the position occupied by Ser_{222}. Activation of these MKKs occurs through dual phosphorylation at these sites because mutagenesis at both positions is needed to obliterate phosphorylation by upstream kinases (Yan *et al.*, 1994; Zanke *et al.*, 1996a).

MKK5 was cloned as a MKK homolog with 45% identity to MKK1, identified with two alternatively spliced forms of 50 and 40 kDa (English *et al.*, 1995; Zhou *et al.*, 1995). Yeast two hybrid screens identified an ERK homolog, ERK5, a 90-kDa protein identified in separate studies as big MAP kinase 1 (BMK1), that contains a Thr–Glu–Tyr motif within its activation lip (Zhou *et al.*, 1995; Lee *et al.*, 1995). Both ERK5 and MKK5 contain sequences outside their consensus kinase core, suggesting the presence of cytoskeletal-binding motifs, and MKK5 is recovered in the particulate fraction of cell extracts. Despite the interaction between these enzymes, no evidence exists that ERK5 can serve as a substrate for MKK5 or any other MKK. To date, ERK5 has no known function; however, it is activated by hydrogen peroxide or sorbitol in smooth muscle cells (Abe *et al.*, 1996), suggesting its regulation by oxidant or osmotic stress.

3. STRESS-ACTIVATED MAP KINASE KINASE KINASES

a. MEKK

MEK kinases (MEKKs) are mammalian homolog of yeast kinases STE11 and Byr2, first discovered through PCR strategies by Lange-Carter *et al.*

(1993). At least nine mammalian MEKKs have been identified so far, including MEKKs 1, 2, 3, and 4, MAPKKK5, Tpl-2, TAK1, ASK1, and NIK.

MEKK1, originally identified as a 5′ truncated mouse clone encoding a 73-kDa polypeptide (Lange-Carter *et al.*, 1993), was later identified in rat as a full length 195-kDa polypeptide consisting of a C-terminal catalytic domain and several N-terminal domains (proline-, cysteine-rich and pleckstrin homology domains) likely to be involved in protein–protein association (Xu *et al.*, 1996). MEKK1 phosphorylates MKK1, MKK2, and MKK4 *in vitro*, although expression studies indicate that JNK/SAPK pathways regulated through MKK4 are preferentially activated over ERK pathways in mammalian cells (Yan *et al.*, 1994; Minden *et al.*, 1994a; Xu *et al.*, 1995; Zanke *et al.*, 1996a). Interestingly, MEKK1 is a poor activator of p38 MAPK *in vitro*, despite the recognition of p38 MAPK by MKK4 *in vitro*; thus it has been proposed that the formation of complexes through interaction domains of various protein kinases are important determinants of specificity (Zanke *et al.*, 1996a). Expressed MEKK1 can be activated by nerve growth factor or phorbol ester through Ras-coupled pathways in PC12 cells (Lange-Carter and Johnson, 1994), and endogeneous proteins immunocross-reactive with MEKK can be activated by a peptide chemoattractant in neutrophils (Avdi *et al.*, 1996). Because MEKK1 is active when expressed in mammalian cells, the mechanisms that govern its regulation are unclear. The ability of MEKK1 to recognize its substrates for phosphorylation might involve cellular targeting, consistent with the observation that the full-length enzyme is enriched within membrane fractions of cell extracts (Xu *et al.*, 1996).

MEKK2 and MEKK3 predict ~70- and 71-kDa polypeptides, respectively, with 94% sequence identity to each other within their kinase domains (vs 50% identity to MEKK1) and greater divergence in their N-terminal domains (Blank *et al.*, 1996). *In vitro*, MEKK2 phosphorylates MKKs 1 and 4, while MEKK3 phosphorylates MKKs 1, 3, and 4 (Ellinger-Ziegelbauer *et al.*, 1997; Blank *et al.*, 1996; Deacon and Blank, 1997), although on expression in mammalian cells, both stimulate JNK/SAPK and ERK, but not p38 MAPK. MEKK4 and MAPKKK5 are respectively 180 and 150 kDa polypeptides with 55% and 40% identity to MEKKs 1, 2, or 3, and stimulate JNK/SAPK, but not ERK or p38 MAPK following cell expression (X. Wang *et al.*, 1996; Gerwins *et al.*, 1997).

Tpl-2 is an oncogene discovered as a locus of provirus insertion in Moloney murine leukemia virus induced rat T-cell lymphomas (Patriotis *et al.*, 1993, 1994) and is >90% identical to the human and mouse Cot oncogene (Miyoshi *et al.*, 1991). Tpl-2 encodes a 51 kDa polypeptide with 32% sequence identity to MEKK1 within its kinase domain and is activated by deletion of 43 amino acids at its C-terminal tail (Patriotis *et al.*, 1994). *In vitro*, Tpl-2 phosphorylates and activates MKK1 and MKK4 (Salmerón *et al.*, 1996) and activates ERK and JNK/SAPK on expression in mammalian cells.

A protein kinase of 64 kDa with 30% sequence identity to the catalytic domain of MEKK1 was revealed in screens for mammalian regulators of yeast STE7. This enzyme was named TGF-β-activated kinase (TAK1) because it is rapidly activated and transcriptionally induced in response to TGF β and bone morphogenic protein 4 (BMP4) and mediates transcriptional activation of TGF-β-responsive enhancers (Yamaguchi *et al.*, 1995b). *In vitro*, TAK1 phosphorylates MKK4 and MKK6 but not MKK1, raising the possibility that it mediates TGF-β signaling through JNK/SAPK pathways (Yamaguchi *et al.*, 1995b; Moriguchi *et al.*, 1996b). Two-hybrid yeast screens identified TAB, a novel protein that interacts with and enhances kinase activity of TAK1 in intact cells (Shibuya *et al.*, 1996). Thus, stress activated kinase cascades are implicated in transcriptional responses to TGF-β signaling.

Apoptosis signal-regulating kinase (ASK1) has 30% sequence similarity to MEKK1 and STE11 in its kinase domain (Ichijo *et al.*, 1997) and is more closely related to MEKKs SSK2 and SSK22 in the yeast HOG1 pathway (Maeda *et al.*, 1995; Section VI,A,3). ASK1 activates MKK3 and MKK4, but not MKK1 *in vitro*, and activates the JNK/SAPK and p38 MAPK pathways, but not ERK, following mammalian cell expression. Endogeneous ASK1 is activated by TNF-α in several cell lines and its overexpression leads to apoptosis (Ichijo *et al.*, 1997). Another MEKK homolog implicated in the cell death response is NF-κB-inducing kinase (NIK), which was identified through its association with TNF receptor-associated factor 2 (TRAF2) adaptor (Malinin *et al.*, 1997). Catalytically inactive NIK mutants block TNF-α or interleukin-1 signaling to NF-κB, and the expression of wild type NIK inhibits cytotoxic responses to TNFα, suggesting a role for this enzyme in NF-κB-mediated resistance to cell death.

b. Mixed Lineage Kinases

A second class of enzymes, distinct from MEKKs, also activate JNK/SAPK and p38 MAPK pathways. Mixed lineage kinases (MLKs) contain leucine zipper domains for protein–protein interactions outside the conserved kinase core. Members of this family include MLK-2 (Dorow *et al.*, 1993; Katoh *et al.*, 1995; Hirai *et al.*, 1997), MLK-3/SPRK/PTK-1 (Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Ing *et al.*, 1994) and DLK/ZPK/MUK (Holzman *et al.*, 1994; Reddy and Pleasure, 1995; Hirai *et al.*, 1996). MLK1 (Dorow *et al.*, 1993) is a related enzyme that is not as thoroughly characterized (Fig. 1).

MLK2 and MLK3/SPRK are 105- and 93-kDa polypeptides, each with a catalytic domain flanked by an SH3 domain on its N-terminal side and by two leucine zipper domains on its C-terminal side. Between the leucine zippers and a proline/glycine-rich C-terminal tail lies a Cdc42/Rac-interactive

binding (CRIB) motif (Ile–Ser–X–Pro–X_{4-6}–His–X–X–His) that enables interaction of these enzymes with Rac1 GTP or Cdc42-GTP (Burbelo *et al*., 1995; Teramoto *et al*., 1996b). *In vitro*, MLK 2 and MLK3/SPRK phosphorylate and activate MKK4. Cell expression of either enzyme triggers both JNK/SAPK and p38 MAPK, and coimmunoprecipitation of MLK3/SPRK with MKK4 or MKK6 has been observed (Rana *et al*., 1996; Tibbles *et al*., 1996), indicating a specific role for these enzymes in stress signaling pathways.

DLK/MUK is a 100-kDa polypeptide that migrates as a 130-kDa polypeptide on SDS PAGE. It contains a catalytic domain flanked on its C-terminal side by a dual leucine zipper motif and proline/glycine rich regions at both N and C termini, although only an incomplete CRIB domain is present (Fan *et al*., 1996). DLK/MUK also activates JNK/SAPK and p38 MAPK when expressed in mammalian cells, and catalytically inactive DLK/MUK can inhibit Ras-dependent JNK/SAPK activation (Hirai *et al*., 1996; Fan *et al*., 1996). Furthermore, the activation of JNK/SAPK is blocked by the expression of a catalytically inactive MEKK1 mutant, indicating that DLK/MUK may directly or indirectly regulate MEKK1 activity (Fan *et al*., 1996). Neither MLK3/SPRK nor DLK/MUK show significant signaling through the MKK/ERK pathway, although MLK-2 activates ERK to an extent comparable to p38 MAPK. However, detailed comparisons of the substrate specificities of these enzymes *in vitro* are still awaited.

4. UPSTREAM REGULATORS OF JNK/SAPK AND p38 MAPK PATHWAYS

a. Rac, Rho, and Cdc42-Regulated Kinases

In analogy to Ras GTP regulation of MKK/ERK, members of the Rho family of small GTPases, including RhoA, Rac1, Rac2, and Cdc42, are positive regulators of JNK/SAPK and p38 MAPK pathways (Coso *et al*., 1995; Minden *et al*., 1995)(Fig. 1). These GTPases are implicated in regulating cytoskeletal reorganization. Rac and Cdc42 regulate cell motility through the formation of lamellipodia and filopodia at the membrane leading edge, whereas Rho is involved in the formation and maintenance of actin stress fibers and focal adhesion plaques as well as the formation of the actin contractile ring at cell division (Nobes and Hall, 1995). Mechanisms linking these proteins to the activation of various MKKs are still unclear but may involve the action of several protein kinases that couple Rac and Cdc42 to stress-activated kinase pathways.

p21Ras-activated kinases (PAKs) are 60- to 70 kDa enzymes that are closely related to yeast STE20 (Leberer *et al*., 1992) within their C-terminal kinase domains; they interact with GTP-Rac and GTP-Cdc42, but not RhoA, through N-terminal CRIB domains. Four mammalian enzymes have been described so far, including PAK1, PAK2, PAK3/βPAK, and γPAK (Manser *et al*., 1994, 1995; Martin *et al*., 1995; Bagrodia *et al*., 1995a; Teo *et al*., 1995). PAK activity and autophosphorylation are stimulated by GTP-Rac or GTP-Cdc42 *in vitro* and *in vivo*, whereas the expression of PAK in mammalian cells leads to activation of JNK/SAPK and p38 MAPK (Bagrodia *et al*., 1995b; Zhang, S. *et al*., 1995; Frost *et al*., 1996). ERK is not a major target for activation by Rac or PAK; however, a potential mechanism for cross regulation is indicated by the ability of PAK to synergize with Raf-1 in stimulating MKK and ERK activity (Frost *et al*., 1996). This may be regulated by the phosphorylation of Ser_{298} on MKK1 by PAK because mutagenesis of this residue to Ala significantly reduces the synergy (Frost *et al*., 1997).

The mechanisms by which PAK activates JNK are not clearly defined, but may involve signaling through MEKKs or MLKs. Dominant negative MLK-3 has been shown to inhibit Cdc42 and Rac signaling to JNK/SAPKs (Teramoto *et al*., 1996b). Alternatively, PAK3 interacts with phospholipase Cγ, and PAKs 1 and 3 interact with the adapter protein, Nck, through SH3 domain interactions with N-terminal proline-rich motifs in PAK (Bagrodia, 1996a; Galisteo *et al*., 1996; Bokoch *et al*., 1996). PAK1 binding to Nck is enhanced by PDGF, leading to elevated Nck phosphorylation, whereas PAK1 activity is enhanced in response to EGF on coexpression with Nck. Thus, Nck might mediate signaling upstream or downstream of PAK.

Although RhoA also regulates JNK/SAPK and p38 MAPK pathways, this does not appear to involve PAK (Teramoto *et al*., 1996a). Instead, RhoA interacts with protein kinase N (PKN), a 120-kDa protein kinase C-related enzyme with N terminal leucine zipper motifs (Mukai and Ono, 1994; Amano *et al*., 1996; Watanabe *et al*., 1996). Rho–PKN interactions lead to the activation of PKN *in vitro* and *in vivo* (Watanabe *et al*., 1996; Amano *et al*., 1996), and PKN translocates from cytosolic to nuclear pools following cell stress (Mukai *et al*., 1996), suggesting that this kinase mediates Rho dependent activation of JNK/SAPK or p38 MAPK. Rac, Rho, and Cdc42 also interact with RhoA-binding kinases α and β (ROKs), 150- to 160-kDa protein kinases with lengthy coiled coil domains, related to human myotonic dystrophy kinase (Leung *et al*., 1995, 1996; Ishizaki *et al*., 1996). These induce actin polymerization on cell expression (Leung *et al*., 1996) and are thus implicated in Rho-dependent cytoskeletal rearrangements. However, experiments introducing point mutations into Rac, Cdc42, and Rho effector domains have shown that the effects of these GTPases on PAK, JNK/SAPK, and p38 MAPK activation are not correlated with their effects on ROK activation, lamellipodia and filopodia extension, or actin stress fiber formation (Lamarche *et al*., 1996; Joneson *et al*., 1996). This suggests that stress-activated kinase pathways are uncoupled from cytoskeletal reorganization events occurring in response to Rac, Cdc42, or Rho.

b. Germinal Center Kinases

Other protein kinases implicated in stress-activated signaling pathways are members of the germinal center kinase (GCK) family, including GCK/Rab8ip, HPK1, MST1, MST2, and SOK1 (Katz *et al.*, 1994; Creasy and Chernoff, 1995a,b; Ren *et al.*, 1996; Hu, M. *et al.*, 1996; Pombo *et al.*, 1996), which are mammalian homologs of yeast STE20 and Sps1 (Friesen *et al.*, 1994). These enzymes are characterized by N-terminal catalytic domains followed by proline-rich regions, leucine-rich regions, and potential PEST sequences (but no Rac interaction motifs). Although none have been found to directly phosphorylate MKKs, on mammalian cell expression these enzymes activate stress-activated kinases, indicating an indirect regulatory role. The 97-kDa GCK is expressed in several tissues, but in lymphoid follicles is restricted to germinal centers, suggesting a role in B cell development (Katz *et al.*, 1994). It is stimulated in response to TNF-α, and its expression leads to MKK4 and JNK/SAPK activation, but not ERK or p38 MAPK activation (Pombo *et al.*, 1995). A closely related mouse homolog, Rab8ip, is found to coimmunoprecipitate and colocalize with Rab8, implicating this enzyme in Golgi vesicle targeting or fusion (Ran *et al.*, 1996)(Fig. 1).

Hematopoetic progenitor kinase 1 (HPK1) is a 97-kDa enzyme that is found predominantly in blood cells and, when overexpressed, selectively activates JNK/SAPK (Kiefer *et al.*, 1996; Hu, M. *et al.*, 1996). The activation of JNK/SAPK by HPK1 is inhibited by dominant negative mutants of MEKK1, MLK-3, or MKK4. HPK1 coprecipitates with and phosphorylates both MEKK1 and MLK-3, suggesting a mode of regulation involving multicomponent complex formation. Mammalian STE20-like kinases (MST1, MST2) and STE20/oxidant stress response kinase (SOK-1) also belong to the GCK family (Creasy and Chernoff, 1995a,b; Pombo *et al.*, 1996). SOK1 is activated by cytokines and oxidants, but does not appear to regulate JNK/SAPK or p38 MAPK.

5. RECEPTORS AND SECOND MESSENGERS INVOLVED IN CELL STRESS

Although JNK/SAPK and p38 MAPK pathways are stimulated by many different types of physical stressors, little is known about the mechanisms for sensing these stimuli. In yeast, HOG1 activation by hyperosmolarity involves at least two osmoreceptors, SLN1 and SHO1 (Maeda *et al.*, 1995; see Section VI,A,3) which may be activated through mechanical membrane distortions. Physical membrane events are also possible in JNK/SAPK activation, based on findings that UV light or hyperosmolarity induces clustering of EGF, TNF-α, or interleukin-1 receptors that might then signal in response to dimerization (Rosette and Karin, 1996). Alternative models invoke DNA damage as common signals induced by UV or γ irradiation, chemical mutagens, and cancer chemotherapeutic agents (e.g., cisplatinum, adriamycin, vinblastine, etoposide). The degree of JNK/SAPK activation has been correlated with the number of DNA lesions induced by UV B or UV C irradiation (Adler *et al.*, 1995), although conflicting results have been obtained regarding the ability of UV to induce JNK/SAPK activation in enucleated cells (Devary *et al.*, 1993; Adler *et al.*, 1995). Reactive oxygen intermediates (ROIs) such as hydroxyl-free radicals are generated by many agents that also activate JNK/SAPK and p38 MAPK, including GTPase deficient mutant Rac(Val12) (Sulciner *et al.*, 1996), suggesting that ROIs provide common signals to JNK/SAPK pathways in response to irradiation, anticancer drugs, or cytotoxic cytokines. This model is supported by the potent activation of JNK/SAPK by oxidants (e.g., H_2O_2) (Lo *et al.*, 1996) and by the inhibitory effect of radical scavengers (e.g., *N*-acetyl-L-cysteine) on JNK/SAPK activation by cell stress (Tao *et al.*, 1996). However, several studies on the effects of oxidants and antioxidants on JNK/SAPK are also conflicting (Yu, R. *et al.*, 1996; Gomez del Arco *et al.*, 1996), and so far a causal role of ROIs in signaling through the kinase pathways has not been definitively established.

Ligand–receptor-mediated pathways implicated in JNK/SAPK and p38 MAPK activation include those involved in inflammatory and/or apoptotic responses, triggered by receptors for TNF-α, IL-1, and Fas ligand as well as the CD14 receptor for lipopolysaccharide. Rac or Cdc42 are activated in response to all of these stimuli and may be common signaling intermediates, although the mechanisms regulating their respective guanine nucleotide exchange factors are unknown. Receptor mediated signaling may also involve several heterotrimeric G_α proteins that have been shown to stimulate JNK/SAPK preferentially over ERK. Activation of JNK/SAPK by GTPase-deficient mutant $G_{\alpha 12}$ or $G_{\alpha 13}$ occurs through mechanisms dependent on Rac and Cdc42 and can be blocked by dominant negative mutant MEKK1 (Prasad *et al.*, 1995; Collins *et al.*, 1996; Voyno-Yasenetskaya *et al.*, 1996). In addition, $G_{\beta\gamma}$ subunits mediate JNK/SAPK signaling through $G_{\alpha i}$ coupled muscarinic receptors (Coso *et al.*, 1996). Ras is implicated in some of these studies, based on inhibitory effects of dominant negative mutant Ras (Prasad *et al.*, 1995; Adler *et al.*, 1996); however, in the majority of cases, stress-activated pathways are Ras independent.

Abelson tyrosine kinase (Abl) is implicated in regulating cell growth, but is also activated by compounds that induce DNA damage, including ionizing radiation (Kharbanda *et al.*, 1995a,b). Both v-Abl and Bcr-Abl activate JNK/SAPK (Renshaw *et al.*, 1996; Raitano *et al.*, 1995). Furthermore, Abl^- cells generated by homologous recombination are unable to activate JNK/SAPK in response to cisplatinum or 1-β-D-arabinofuranosylcytosine (AraC), although cells still respond to UV light and alkylating agents, implying the existence of convergent stress-signaling pathways (Kharbanda *et al.*, 1995b; Pandey *et al.*, 1996). These findings indicate an upstream regu-

latory role for c-Abl, most likely mediated through Rac, because dominant negative mutant Rac inhibits v-Abl-induced JNK/SAPK activation (Renshaw *et al.*, 1996). Another tyrosine kinase implicated in JNK/SAPK activation is Pyk2, which is activated in response to TNF-α, UV irradiation, and hyperosmolarity, and activates JNK/SAPK on overexpression (Tokiwa *et al.*, 1996). A calcium dependent tyrosine kinase, partially purified and found to be related to Pyk2, was stimulated by thapsigargin and angiotensin II under conditions resulting in substantial JNK activation and minimal ERK activation (Yu, H. *et al.*, 1996).

Lipid second messengers are implicated in stress-activated kinase signaling. In response to TNF-α, interleukin 1, Fas ligand, or ionizing radiation, ceramide levels increase as a result of elevated sphingomyelinase activity. Activation of JNK/SAPK is also observed on direct treatment of cells with cell-permeable ceramide analogs (Westwick *et al*., 1995; Pyne *et al.*, 1996; Welsh, 1996; Coroneos *et al.*, 1996). Lipid products of PI3′K may also be involved in signaling to JNK/SAPK kinases. Elevation of Rac-GTP and activation of JNK/SAPK are inhibited by wortmannin under some conditions (Hawkins *et al.*, 1995; Ishizuka *et al.*, 1996), and a constitutively active mutant of PI3′K stimulates JNK/SAPK (Klippel *et al.*, 1996). Thus, in addition to other Rac or Rho like morphological responses (Reif *et al.*, 1996), JNK/SAPK may be regulated by products of PI3′K through activation of Rac. Further evidence for a role of PI3′K in JNK/SAPK signaling is provided by studies in cells derived from patients with ataxia telangiectasia (A-T), a deficiency in a PI3′K like enzyme, where ionizing radiation (but not UV or anisomycin treatment) failed to activate JNK/SAPK (Shafman *et al.*, 1995). However, ionizing radiation induces an association between JNK/SAPK and the p85 subunit of PI3′K, mediated through Grb2 (Kharbanda *et al.*, 1995c). Under the latter conditions, wortmannin stimulated JNK/SAPK activity, suggesting inhibitory effects of PI3′K on JNK/SAPK activation.

6. JNK/SAPK AND p38 MAPK PATHWAYS IN APOPTOSIS

Many agents that activate JNK/SAPK are cytotoxic, suggesting that this kinase pathway positively regulates cell death. In blood cells, cytokine stimulated apoptosis is mediated through ceramide synthesis and can be induced by ceramide uptake into cells and antagonized by sphingosine 1-phosphate (Obeid *et al.*, 1993; Cuvillier *et al.*, 1996). A causal role for JNK/SAPK in cell death is suggested by the finding that ceramide-induced apoptosis in U937 cells and UVC or γ radiation-induced apoptosis in T cells can be inhibited by the expression of dominant negative mutant MKK4 (Verjeij *et al.*, 1996; Chen, Y. *et al.*, 1996a). Furthermore, ERK is activated by sphingosine 1 phosphate and the cytoprotective effect of this metabolite requires ERK

activity because it is suppressed by PD98059 (Cuvillier *et al.*, 1996; Pyne *et al.*, 1996). Similar results have been reported in model systems for neuronal cell apoptosis. PC12 cells differentiated with nerve growth factor (NGF) undergo apoptosis following NGF withdrawal. Cell death is accompanied by the activation of JNK/SAPK and p38 MAPK and appears to be dependent on these pathways, because expression of dominant negative mutant MKK3 or MKK4 promotes cell survival (Xia *et al.*, 1995). PC12 cell survival is also enhanced by the expression of constitutively active MKK1 (Xia *et al.*, 1995). Thus, in neuronal and blood cells, relative levels of signaling through ERK vs JNK/SAPK or p38 MAPK appear to regulate the choice between cell survival vs death. Consistent with this model, a role of JNK/SAPK in epithelial cell apoptosis induced by integrin-extracellular matrix disruption (anoikis) is blocked by dominant negative mutant MKK4 (Frisch *et al.*, 1996).

Although JNK/SAPK activation is correlated with apoptosis induced by ceramides and UV and γ irradiation, the same may not be true for TNF-α- or Fas-induced apoptosis. Thus, JNK/SAPK activation was inhibited by dominant negative MEKK1 or MKK4 in response to TNF-α or Fas, respectively, with no effect on ligand-induced cell death (Liu *et al.*, 1996; Lenczowski *et al.*, 1997). Correspondingly, apoptosis induced by TNF-α or Fas was inhibited by mutagenesis of the Fas/TNFR1 receptor interacting proteins, RIP or FADD, or with interleukin-1 converting enzyme (ICE) protease inhibitors, with no reduction in JNK activation. Although the p38 MAPK inhibitor, SB203580, had no effect on TNF-α- or Fas-mediated apoptosis (Juo *et al.*, 1996; Bayaert *et al.*, 1996), expression of active MKK3 or MKK6 augmented the Fas-dependent cell death response (Juo *et al.*, 1996; Huang *et al.*, 1997), indicating a potential involvement of MKK3/6 through a p38 MAPK-independent mechanism. Furthermore, whereas JNK/SAPK and p38 MAPK are involved in some but not all apoptotic responses, activation of JNK/SAPK during anoikis and Fas-dependent activation of p38MAPK in T cells can be inhibited by the CrmA protease inhibitor (Frisch *et al.*, 1996; Juo *et al.*, 1996), providing evidence for a positive feedback between stress-activated kinases and ICE proteases.

III. REGULATION OF MAPK PATHWAYS BY PROTEIN PHOSPHATASES

Protein phosphatases are catagorized into two general groups, the protein phosphatases (PPs) and the protein tyrosine phosphatases (PTPs). Attenuation of signaling in the various MAPK pathways is controlled by members of both these families (Table I). The PPs specifically hydrolyze serine/threo-

Table I Regulatory Phosphatases in MAPK Pathways

Phosphatase[a]	Target	Localization	Reference
Mammalian			
PPs			
PP2A	ERK and MEK	Cytosol (mainly)	Anderson *et al.* (1990), Gomez and Cohen (1991), Sontag *et al.* (1993), Alessi *et al.* (1995), Cohen (1989)
DSPs			
MKP-1 (CL100, ERP,3CH134, hVH1)	ERK, JNK, p38	Nucleus	Sun *et al.* (1993), Alessi *et al.* (1993), Noguchi *et al.* (1993), Zheng and Guan (1993), Brondello *et al.* (1995), Chu *et al.* (1996)
PAC-1	ERK and p38	Nucleus	Rohan *et al.* (1993), Ward *et al.* (1994), Chu *et al.* (1996)
MKP-2 (hVH2, TYP1)	ERK, JNK, p38	Nucleus	Guan and Butch (1995), Misra-Press *et al.* (1995), Kung *et al.* (1995), Chu *et al.* (1996), Brondello *et al.* (1997)
MKP-3 (rVH6, PYST1)	ERK	Cytosol	Mourey *et al.* (1996), Muda *et al.* (1996b), Groom *et al.* (1996), Muda *et al.* (1996a)
hVH-3 (B23)	ERK	Nucleus	Kwak and Dixon (1995), Ishibashi *et al.* (1994)
M3/6	JNK and p38	Cell type dependent	Theodosiou *et al.* (1996), Muda *et al.* (1996a)
hVH-5	ERK		Martell *et al.* (1995)
VHR	ERK		Ishibashi *et al.* (1992), Ishibashi *et al.* (1994)
MKP-X (PYST2)			Groom *et al.* (1996), Muda *et al.* (1996b)
MKP-4	ERK, JNK, p38		Muda *et al.* (1997)
Saccharomyces cerevisiae			
PPs			
PTC1,2	(HOG1 and PBS2)[b]		Maeda *et al.* (1994)
PTPs			
PTP2,3	HOG1		Maeda *et al.* (1994), Jacoby *et al.* (1997), Wurgler Murphy *et al.* (1997)
DSPs			
MSG5	FUS3 and MPK1		Doi *et al.* (1994), Watanabe *et al.* (1995)

(*continues*)

Table I (*continued*)

Phosphatase[a]	Target	Localization	Reference
Schizosaccharomyces pombe			
PPs			
Ptc1,2,3	(Spc1 and Wis1)[b]		Shiozaki and Russell (1995b)
PTPs			
Pyp1,2	Spc1		Shiozaki and Russell (1995a), Millar *et al.* (1995), Degols *et al.* (1996)
DSPs			
Dsp1	Pmk1		Toda *et al.* (1996)

[a]PPs, Ser/Thr protein phosphatases; PTPs, protein tyrosine phosphatases; DSPs, dual specificity phosphatases.

nine phosphoesters and include PP1, PP2A, PP2B, and PP2C (Wera and Hemmings, 1995). Hydrolysis of tyrosine phosphoesters is catalyzed by the PTPs, which include a subfamily of dual specificity phosphatases (DSPs) capable of hydrolyzing both phosphotyrosine and phosphoserine/threonine (Fauman and Saper, 1996).

Although PPs and PTPs have similar functions, their catalytic mechanisms and structures are different (reviewed by Denu *et al.*, 1996a; Barford, 1996). The crystal structures of PP1 (Goldberg *et al.*, 1995; Egloff *et al.*, 1995), PP2B (Griffith *et al.*, 1995; Kissinger *et al.*, 1995), and PP2C (Das *et al.*, 1997) have been determined, revealing a central β–α–β–α–β motif containing a dinuclear metal ion center at the active site. Based upon structural data, a single-step catalytic mechanism has been proposed in which metal-activated water directly attacks the phosphorous center of the substrate. In contrast, PTPs do not require metal ions for catalysis, but utilize a catalytic cysteine present in the conserved active site sequence motif: His–Cys–X–X–Gly–X–X–Arg–Ser/Thr. Catalysis occurs in a two-step mechanism in which a phosphoenzyme intermediate forms at the cysteine followed by hydrolysis by water to restore the enzyme (Denu and Dixon, 1995a, 1996b). Comparison of the PTP1B structure (Jia *et al.*, 1995) with that of the dual specificity phosphatase VHR (Yuvaniyama *et al.*, 1996) suggests that the depth of the active site pocket determines substrate specificity. The shallow active site pocket of VHR accommodates phosphorylated serine, threonine, and tyrosine, whereas the deeper active site of PTP1B favors the longer phosphotyrosine side chain (Yuvaniyama *et al.*, 1996).

A. Dual Specificity Phosphatases

Dual specificity phosphatases constitute the largest family of phosphatases capable of regulating MAPKs in mammalian systems. DSPs that modulate MAPK pathways are homologous, especially in their catalytic domains; each is related to the prototypical VH1 dual specificity phosphatase of vaccinia virus (Guan *et al.*, 1991). This group of MAPK phosphatases (MKPs) includes the following members: MKP-1 (also named CL100, 3CH134, hVH-1, or ERP), MKP-2 (hVH-2 or TYP-1), MKP-3 (rVH-6 or PYST1), MKP-4, MKP-X (PYST2), PAC1, hVH-3 (B23), M3/6, hVH-5, and VHR (Table I). Although similar in function, members of this family vary in substrate specificity, localization, regulation of gene expression, and tissue distribution.

Because MAPKs require both threonine and tyrosine phosphorylation for full activity, dual specificity MKPs are in a unique position to regulate MAPK signal transduction cascades. MKP-1, the first of the MKPs to be characterized, inactivates ERK2 *in vitro* by concomitant dephosphorylation of phosphothreonine and phosphotyrosine residues (Charles *et al.*, 1993; Alessi *et al.*, 1993; Zheng and Guan, 1993b). ERKs are also effectively inactivated by MKP-1 *in vivo* (Sun *et al.*, 1993; Duff *et al.*, 1995). Similarly, PAC1, MKP-2, and MKP-3 inactivate ERK1/2 both *in vitro* and *in vivo*, whereas hVH-3, hVH-5, and VHR do so only *in vitro* (Table I). The stress-activated kinases JNK/SAPK and p38 MAPK are also substrates for MKP-1 and MKP-2, whereas PAC1 recognizes only p38 MAPK (Chu, Y., *et al.*, 1996; Brondello *et al.*, 1997). However, JNK/SAPK inactivation requires neither MKP-1 nor MKP-2 *in vivo*, and ERK is a better MKP-1 substrate than JNK/SAPK (Sun *et al.*, 1994; Brondello *et al.*, 1997). MKP-3 is specific for ERKs1 and 2, whereas M3/6 selectively inactivates JNK/SAPK and p38 MAPK (Muda *et al.*, 1996a; Groom *et al.*, 1996). The substrate specificities of individual MKPs imply their differential ability to regulate various MAPK pathways. Indeed, MKP-3 blocks growth factor stimulation of ERK1 *in vivo*, but not stress-induced activation of JNK1/SAPK or p38 MAPK (Groom *et al.*, 1996). Conversely, M3/6 is restricted to JNK1/SAPK and p38 MAPK and has no effect on EGF stimulation of ERK1 (Muda *et al.*, 1996a). Overexpressed MKP-1 is able to block both signaling pathways (Sun *et al.*, 1993; Liu, 1995).

In general, MKPs localize within nuclei, providing a mechanism for the inactivation of nuclear MAPK (Table I). MKP-1 is exclusively nuclear in both quiescent and stimulated cells (Brondello *et al.*, 1995; Lewis *et al.*, 1995), as are PAC1, MKP-2, and hVH-3 (Rohan *et al.*, 1993; Guan and Butch, 1995; King *et al.*, 1995; Kwak and Dixon, 1995). In contrast, microinjected M3/6 shows localization dependent on cell type, being nuclear in Swiss 3T3 cells, but cytoplasmic in MDCK and PC12 cells (Theodosiou *et al.*, 1996). MKP-3 and MPK-4 are excluded from the nucleus and thus appear to be a regulator of cytoplasmic ERK1 and ERK2 (Groom *et al.*, 1996; Mourey *et al.*, 1996; Muda *et al.*, 1996b, 1997). Cytoplasmic vs nuclear regulation of MAPK signaling functionally distinguishes MKP-3 and -4 from the other MKPs.

Most MKPs are transcriptionally upregulated in response to mitogenic and stress stimuli, although in different ways. MKP-1 was originally identified as an immediate early gene responsive to oxidative stress and heat shock, as well as serum stimulation (Lau and Nathans, 1985; Keyse and Emslie, 1992). UV light and anisomycin treatment also rapidly enhance MKP-1 mRNA levels (Liu *et al.*, 1995; Bokemeyer *et al.*, 1996). PAC1 is a mitogen-induced early response gene that is predominantly expressed in hematopoietic tissues (Rohan *et al.*, 1993). Similarly, induction of hVH-5 by NGF and insulin is characteristic of an immediate early gene (Martell *et al.*, 1995). In contrast, the other MKPs differ from MKP-1 in their magnitude and kinetics of mRNA expression. hVH-3 is induced neither by oxidative stress nor UV light and the time course of its mRNA expression in response to insulin is prolonged (Kwak and Dixon, 1995; Groom *et al.*, 1996). The upregulation of MKP-2 message in response to mitogenic stimuli is delayed and less pronounced compared to MKP-1. In addition, MKP-2 transcription is not induced by oxidative stress or heat shock (King *et al.*, 1995; Guan and Butch, 1995; Misra-Press *et al.*, 1995). VHR is unresponsive to extracellular stimuli, including oxidative stress or growth factor treatment (Kwak and Dixon, 1995). The cytosolic MKP-3 is not an immediate early gene; its message expression is weakly induced by serum and is unaffected by heat shock, oxidative stress, or UV light (Groom *et al.*, 1996). In PC12 cells, NGF causes a robust upregulation of MKP-3, whereas in MM14 muscle cells, bFGF causes a similar induction (Muda *et al.*, 1996b; Mourey *et al.*, 1996). bFGF withdrawal from MM14 cells decreases MKP-3 expression, resulting in a subsequent increase in ERK activity, concomitant with differentiation (Mourey *et al.*, 1996).

MKP mRNA expression is regulated by mitogenic and stress-activated MAPK pathways. MKP-1 promoters contain Sp1, NF1-like, AP2, AP1, E-box, and CRE enhancer sites as well as a TATA basal promoter (Noguchi *et al.*, 1993; Kwak *et al.*, 1994). AP1 promoters are known to be regulated by ERK or JNK/SAPK phosphorylation of c-Jun and CRE promoters can be regulated by JNK/SAPK or p38 MAPK phosphorylation of ATF2 (see Section IV,A). Indeed, MKP-1 appears to be regulated by both ERK1/2 and JNK/SAPK in a cell type-specific fashion. In NIH3T3 cells, activation of the JNK/SAPK pathway induces MKP-1 gene expression whereas selective stimulation of the ERK pathway inhibits MKP-1 transcription (Bokemeyer *et al.*, 1996). Thus, MKP-1 mediates cross-talk between ERK and JNK/SAPK pathways in this system because JNK/SAPK induces MKP-1, which in turn inactivates ERK (Bokemeyer *et al.*, 1996). In contrast, in CCL39 fibroblasts, activation of ERK induces MKP-1 and MKP-2 expression, forming a negative feedback loop, whereas agonists that activate JNK have no effect on MKP-

1 or MKP-2 (Brondello *et al.*, 1997). The incongruous results in these studies necessitate further investigation into the role of MAPKs in MKP-1 regulation. Similar to MKP-1, the PAC1 promoter contains an AP2 site and an E box element. PAC1 is positively regulated by the ERK pathway in mouse hematopoietic cells, constituting another inhibitory feedback loop (Grumont *et al.*, 1996). Activation of yeast pheromone response and stress-activated MAPK pathways is also attenuated by the feedback induction of PTPs and DSPs (see Section VI). Thus, it is apparent that negative feedback loops are a conserved mechanism for MAPK pathway downregulation.

B. Serine/Threonine Phosphatases

Dual specificity MKPs are not the exclusive regulators of MAPK pathways, as serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) negatively regulate both MKKs and MAPKs. PP1 and PP2A inactivate ERK1 and ERK2 and MKK1 and MKK2 *in vitro* by dephosphorylation of regulatory Ser/Thr residues within their activation lip (Anderson *et al.*, 1990; Ahn *et al.*, 1991,1993; Gomez and Cohen, 1991; Nakielny *et al.*, 1992). In addition, treatment of cells with okadaic acid, a potent inhibitor of PP1 and PP2A, activates the ERK pathway (Haystead *et al.*, 1990; Gotoh *et al.*, 1990b; Casillas *et al.*, 1993). Overexpression of SV40 small T antigen, which interacts with and inhibits PP2A, stimulates MKK1 and ERK activity while having no effect on Raf-1 activity in CV-1 cells (Sontag *et al.*, 1993). In PC12, PAE, or 3T3-L1 cells, the rapid (15 min) inactivation of ERK following growth factor stimulation occurs independently of MKP-1 expression (Wu *et al.*, 1994; Alessi *et al.*, 1995b). In these cells, PP2A is the major phosphatase acting on ERK and MKK1, functioning coordinately with an unidentified Tyr_{185}-specific phosphatase.(Alessi *et al.*, 1995b). PP2A is negatively regulated by phosphorylation of its catalytic subunit (Tyr_{307}) by receptor and nonreceptor tyrosine kinases (Chen, J. *et al.*, 1992, 1994). Furthermore, phorbol ester stimulation of ventricular cardiomyocytes transiently suppresses PP2A activity, correlating with phosphorylation of the PP2A catalytic subunit (Quintaje *et al.*, 1996). This temporary decrease in PP2A activity is accompanied by a transient increase in cytosolic ERK activity, indicating that PP2A is a physiological regulator of ERK.

C. Protein Tyrosine Phosphatases

Tyrosine-specific phosphatases also attenuate signaling in MAPK modules. *S. cerevisiae* and *S. pombe* utilize PTPs in coordination with PP2C-related PTCs to regulate stress response pathways (Table I). In mammals, selective

dephosphorylation of Tyr_{185} on ERK2 by the transmembrane PTP, CD45, is completely inactivating (Anderson *et al.*, 1990). In general, a well defined PTP that regulates ERK physiologically has yet to be reported in vertebrate systems, although one ERK-inactivating PTP has been purified to near homogeneity from *Xenopus* eggs (Sarcevic, 1993). Such an enzyme would probably act in conjunction with PP2A, efficiently dephosphorylating Tyr_{185} following the rate-limiting removal of Thr_{183} phosphoester by PP2A (Alessi *et al.*, 1995b). The advantage of MAPK regulation by dual specificity phosphatases vs tyrosine specific phosphatases probably resides in substrate specificity. For example, dual specificity VHR preferentially recognizes diphosphorylated substrate in an ordered mechanism that entails rapid removal of phosphotyrosine followed by slow hydrolysis of phosphothreonine (Denu *et al.*, 1995b). Thus, DSPs do not require the initial action of a serine/threonine phosphatase in order to efficiently dephosphorylate phosphotyrosine.

IV. CELLULAR SUBSTRATES OF MAP KINASES

In vitro, the only targets for MKKs that have been identified are MAP kinases, therefore, specificity of downstream signaling in MKK/MAPK cascades appears to be controlled at the level of ERK, JNK/SAPK, and p38 MAPK. ERK favors substrates containing Pro residues at the P+1 position, with secondary preference for Pro at the P-2 position (Alvarez *et al.*, 1991; Davis, 1993). JNK/SAPK and p38 MAPK also recognize Pro at P+1, although the sequence determinants that confer differential recognition by ERK, JNK, or p38 MAPK have not been reported. *In vitro* targets for MAP kinases include nuclear transcription factors, metabolic enzymes, cytoskeletal proteins, and other signaling components (Table II). Many of these also appear to be physiological targets, based on peptide map comparisons between *in vitro* vs *in vivo* phosphorylation sites. Important reagents have become available that enable further correlation between kinase activation and substrate phosphorylation in response to various stimuli.

Constitutively active mutants of Raf and MKKs have been expressed or microinjected into cells to activate signaling through various MAP kinase pathways. A particularly useful construct fuses the N-terminally truncated Raf-1 catalytic domain to the steroid-binding domain of the estrogen receptor (McCarthy *et al.*, 1995). This chimera can be activated within minutes of adding estradiol to cells and has been used to measure effects of acute signaling through the Raf/MKK/ERK pathway (Pumiglia and Decker, 1997; Bianchini *et al.*, 1997). Active MKK1 and MKK2 mutants have been designed with activities ranging from 10-fold over basal to those comparable

Table II Substrates for MAP Kinases and MAPKAP Kinases

Substrate	Function	Sequence	Reference
A. ERK 1/2 substrates			
1. Protein kinases			
Rsk-1, Rsk-2		$LLMT_{577}PCYT_{574}ANFV$	Sutherland *et al.* (1993a)
Rsk-3			Y. ZHao *et al.* (1995)
MAPKAP kinase 2		$PQPPT_{25}PALP$	Ben-Levy *et al.* (1995)
		$NSLTT_{222}PCY$	
		$AIS_{272}PGMK$	
		$VPQT_{334}PLHTSR$	
MAPKAP kinase 3			Ludwig *et al.* (1996)
Mnk1, Mnk2			Waskiewicz *et al.* (1997)
2. Transcription factors			
$p62^{TCF}$/Elk-2	SRF activation	$STLS_{383}PIAPRS_{389}PAK$	Cavigelli *et al.* (1995)
SAP-1a, SAP-2	SRF activation	$STLS_{383}PVAPLS_{389}PAR$	Price *et al.* (1995)
ER81	Ets activation		Janknecht (1996)
Pointed P2 (*Drosophila*)	Ets repression	$PLT_{151}PG$	Brunner *et al.* (1994a)
Yan (*Drosophila*)	Ets repression		Rebay and Rubin (1995)
ERF	Ets repression	$GGPLT_{526}PRRV$	Sgouras *et al.* (1995)
PPAR-γ	Suppression adipogenesis	$PAS_{112}P$	Hu *et al.* (1996)
Estrogen receptor	Receptor activation	$PPPQLS_{118}PF$	Kato *et al.* (1995)
c-Fos	Fos stabilization	$DSLSS_{374}PTLL$	R. Chen *et al.* (1996)
Fra-1, Fra-2			Gruda *et al.* (1994)
c-Jun	Inhibition DNA binding	$TPPLS_{246}PIDME$	Alvarez *et al.* (1991)
JUN (*Drosophila*)	AP-1 activation	$VINS_{82}PD$, $TVNT_{92}PD$	Peverali *et al.* (1996)
c-Myc	Myc activation	$LPPTS_{62}PSRR$	Alvarez *et al.* (1991)
N-Myc	Myc repression	$PLS_{51}PS$	Manabe *et al.* (1996)
HSF-1	Heat shock response	$PPSPPQS_{307}PR$	B. Chu *et al.* (1996)
GATA-2	Hematopoietic transcription		Towatari *et al.* (1995)
TAL-1/SCL	TAL-1 activation	$MVQLS_{122}PPAL$	Wadman *et al.* (1994)
NF-IL6	Interleukin-6 signaling	$SSPPGT_{235}PSP$	Nakajima *et al.* (1993)
NTF-1/Elf-1 (*Drosophila*)	Torso response element		Liaw *et al.* (1995)
Tristetraprolin	Early response gene	$PLS_{220}PS$	Taylor *et al.* (1995)
AML1	Acute myeloid leukemia	$QIQPS_{249}PPWS$	Tanka *et al.* (1996)
		$GSIAS_{266}PSVH$	
p53	Checkpoint control	$TET_{73}PGP$, $PAT_{81}PW$	Milne *et al.* (1994)
3. Signaling components			
SOS1	p21Ras activation	$GPRS_{1132}AS$	Corbalan-Garcia *et al.* (1996)
		$AESS_{1167}PS$	
		$HLDS_{1178}PPA$	
		$PRYS_{1197}IS$	
EGF receptor	EGF signaling	$VEPLT_{669}PSGEA$	Northwood *et al.* (1991)
Phospholipase A2	PLA_2 activation	$SYPLS_{505}PLSD$	Lin *et al.* (1993)
HSPDE4B2B	cAMP phosphodiesterase	$IPQS_{487}PS_{489}PPL$	Lenhard *et al.* (1996)
Rab4	Glucose transport	$QLRS_{196}PRTT$	Cormont *et al.* (1994)
Inhibitor-2	PP1 activation	$PST_{72}PY$	Q. Wang *et al.* (1995)
PTP-2C	PTPase inhibition		Peraldi *et al.* (1994)
4. Cytoskeletal proteins			
MAP-2	Microtubule association		Ray and Sturgill (1987)
Caldesmon	Smooth muscle contraction	$NKS_{739}PAPK$	Adam and Hathaway (1993)
		$VTS_{759}PTK$	
Dystrophin	Cytoskeleton organization		Shemanko *et al.* (1995)
Synapsin I	Neurotransmitter release	$PVAS_{62}PAAPS_{67}PGS$	Jovanovic *et al.* (1996)
		$PPAS_{549}PSPQ$	
Tau	Microtubule association	$YSS_{199}PGS_{202}P$	Drewes *et al.* (1992)
5. Other targets			
Tyrosine hydroxylase	Neurotransmitter synthesis	$AIMS_{31}PREK$	Haycock *et al.* (1992)
Connexin-43	Gap junction communication	$PLS_{255}PSK$	Warn-Cramer *et al.* (1996)
		$PLS_{279}PMS_{282}PP$	
Stathmin	Microtubule polymerization	$ILS_{25}PRS$, $PLS_{38}PP$	Leighton *et al.* (1993)
Oncoprotein M		$LILS_{25}PRSK$	Marklund *et al.* (1993)
Topoisomerase II α	DNA supercoiling	$LPS_{1212}PRG$	Wells and Hickson (1995)

(continues)

to phosphorylated enzyme (Brunet *et al.*, 1994b; Huang and Erikson, 1994; Mansour *et al.*, 1994; Cowley *et al.*, 1994; Mansour *et al.*, 1996b). Constitutively active mutants of MKK3 and MKK6 have also been developed by the substitution of regulatory phosphorylation sites with acidic amino acids (Xia *et al.*, 1995; Raingeaud *et al.*, 1996). Finally, a constitutively active mutant of ERK mutation ("sevenmaker," D319N) was identified by its gain-of-function phenotype in *Drosophila* eye development (Brunner *et al.*, 1994b). Although the mutant ERK appears to be regulated normally by phosphorylation, its elevated activity is conferred by its greater resistance to dephosphorylation and inactivation by several protein phosphatases (Brott *et al.*, 1994; Chu, Y. *et al.*, 1996; Oellers and Hafen, 1996). This mutant has been used with variable success in mammalian systems due to its low level of activity (three- to fourfold).

Several inhibitors have been useful for defining the involvement of various MKKs or MAPKs in signaling responses. Catalytically inactive mutants of MKKs 1–4 and 6, ERKs 1 and 2, JNK/SAPK, and p38 MAPK have been reported to work as dominant negative regulators of signaling, presumably through nonproductive interactions with upstream activators or downstream targets (Alawi *et al.*, 1993; Frost *et al.*, 1994; Zanke *et al.*, 1996; Raingeaud *et al.*, 1996). Inhibition of signaling has also been achieved using full-length antisense transcripts or antisense oligonucleotides directed toward Raf-1 or ERK (Pages *et al.*, 1993; Sale *et al.*, 1995; Muthalif *et al.*, 1996). PD98059 is a cell-permeable inhibitor selective for MKK1 and MKK2, which blocks MKK activation at concentrations ranging from 10 to 100 μM (Dudley *et al.*, 1995; Alessi *et al.*, 1995a). *In vitro*, the IC_{50} for PD98059 ranges from 2 μM (for MKK1) to 50 μM (for MKK2). Antiinflammatory drugs, SB203580 and SB202190, are inhibitors of p38 MAPK α and β (but not γ or δ), with IC_{50} 1 μM. (Lee *et al.*, 1994; Bayaert *et al.*, 1996). Using these reagents, the involvement of different MKKs or MAPKs in various cellular responses can be established by correlating dose–response curves for signaling inhibition vs kinase inhibition.

A. Protein Kinase Substrates for MAPKs

Five protein kinases can be activated by ERK phosphorylation and thus mediate MKK/ERK signaling, referred to as MAPK activated protein (MAPKAP) kinases 1, 2, and 3 and Mnks 1 and 2. MAPKAP kinase-1 α and β were first described as ribosomal S6 kinases, pp90 Rsk-1 and Rsk-2 (Sturgill *et al.*, 1988), which are activated through phosphorylation at two threonine residues within the activation lip (Sutherland *et al.*, 1993a; Grove *et al.*, 1993). However, although ribosomal protein S6 was the substrate first used to identify Rsk activity, S6 appears to be a physiological target not of Rsk,

Table II. *Continued*

Substrate	Function	Sequence	Reference
B. JNK/SAPK substrates			
MAPKAP kinase 3			
$p62^{TCF}$/Elk-1	SRF activation	$STTS_{383}PLAPRS_{389}PAK$	Tudmor *et al.*, 1996
P53		$VTET_{-1}PQP$	(Jayaraman *et al.*, 1995
		$APAT_{81}PW$	Milne *et al.*, 1995
Neurofilament H	Neurofilament polymerization	KSPXE repeats	(Hasson *et al.*, 1996
c-Jun	AP-1 activation	$LLTS_{63}PDVGLLKLAS_{-1}PLL$	Pulverer *et al.*, 1991
C. p38 MAPK substrates			
MAPKAP kinase 2		$LLT_{205}PCVLPQT_{31}-PL$	Engel *et al.*, 1995a
MAPKAP kinase 3			Ludwig *et al.*, 1996
ATF2	ATF2 activation	$DQT_{69}PT_{-1}PT$	(Jaspers *et al.*, 1995
(CHOP(GADD153)	C/EBP activation	$RTSQS_{-9}PHS_{-1}PDSS$	Wang and Ron 1996
Phospholipase A2			Framet *et al.*, 1996)
Max	Max activation		Zervos *et al.*, 1995
D. RSK1,2 substrates			
GSK-3 α		$RARTSS_{21}FAEP$	Sutherland and Cohen 1994
GSK-3 β		RPRTTSAFAES	Stambolic and Woodgett 1994
Glycogen targetting subunit	PP1 activation	$RRGS_{46}ESS$	Dent *et al.*, 1990)
L1	Neural cell adhesion	$KCGKYS_{1152}VK$	Wong *et al.*, 1996
Tyrosine hydroxylase	DOPA synthesis	$RRQS_{40}LIE$	Sutherland *et al.*, 1993b)
Nur77	Transcriptional regulation		(Chen *et al.*, 1992)
c-Fos	Fos stabilization	$AHRRGS_{362}SSX$	R. Chen *et al.*, 1996)
SRF	SRF activation	$RSLS_{103}ENI$	Chen *et al.*, 1992)
CREB	CREB activation	$RRPS_{133}YR$	Xing *et al.*, 1996)
CBP	Transcriptional coactivation		Sakaitani *et al.*, 1996
E. MAPKAP kinase 2 substrates			
$hsp27$	Actin polymerization	$RQLS_{82}SSGV$	Stokoe *et al.*, 1992b
Tyrosine hydroxylase	DOPA synthesis	$RRAVS_{19}ELD$, $RRQS_{40}LIE$	Sutherland *et al.*, 1993b
LSP-1	Actin binding	$KSNS_{200}VKK$, $MAS_{252}TKT$	Huang *et al.*, 1997
F. MAPKAP kinase 3 substrate			
$Hsp27$	Actin polymerization	$RQLS_{82}SSGV$	Clifton *et al.*, 1996

but of p70 S6 kinase (Chung *et al.*, 1992). Another homolog, Rsk-3, is phosphorylated but not activated by ERK2, suggesting that ERK may be needed but is not sufficient for activation (Zhao, Y. *et al.*, 1995). MAPKAP kinase-1 isoforms each have two consensus kinase catalytic domains, both which are functional, with ERK phosphorylation elevating the activity of the C-terminal domain (Fisher and Blenis, 1996). These enzymes target several substrates for phosphorylation, with specificity for Arg at position P-3 (Stokoe *et al.*, 1993). The glycogen-associated targeting subunit of PP1 is phosphorylated by ISPK, a protein kinase with properties similar to MAPKAP kinase-1, conferring activation of PP1 in response to insulin (Lavionne *et al.*, 1991; Begum, 1995). Glycogen synthase kinase-3, a protein kinase involved in glycogen synthesis as well as cell fate determination, is inactivated *in vitro* and in intact cells on phosphorylation by MAPKAP kinase-1α (Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Eldar Finkelman *et al.*, 1995). Some substrates for MAPKAP kinase 1 are involved in transcription, including cAMP response element-binding protein (CREB) (Ginty *et al.*, 1994; Xing *et al.*, 1996), CREB binding protein (CBP) (Nakajima *et al.*, 1996), c-fos, Nur77, and serum response factor (Chen, R. *et al.*, 1992, 1996). Thus activation of Rsk/MAPKAP kinase-1 by ERK provides a means of gene regulation. Genetic lesions in MAPKAP kinase-1β are implicated in Coffin–Lowry syndrome (Trivier *et al.*, 1996), indicating a role for Rsk in human developmental diseases.

MAPKAP kinase-2 is a 40-kDa enzyme, which in addition to a single catalytic domain contains a C-terminal autoinhibitory domain (Zu *et al.*, 1995) and an N-terminal proline-rich region that interacts with SH3 domain-containing proteins (Engel *et al.*, 1993; Plath *et al.*, 1994). Activation occurs through phosphorylation within the catalytic as well as the autoinhibitory domain (Ben-Levy *et al.*, 1995; Engel *et al.*, 1995a). MAPKAP kinase-2 is phosphorylated by both p38 MAPK and ERK, although p38 MAPK is probably the more important physiological regulator because many growth factors that strongly activate ERK do not activate MAPKAP kinase-2 (Stokoe *et al.*, 1992a; Rouse *et al.*, 1994). Once activated, MAPKAP kinase-2 recognizes substrate specificity determinants of Arg at P-3 and hydrophobic residues at P-5 (Stokoe *et al.*, 1993). MAPKAP kinase-3 (also called chromosome 3p kinase, 3pK) is a 42-kDa enzyme identified in a genomic locus frequently disrupted in small cell lung cancers (Sithanandam *et al.*, 1996). *In vitro*, MAPKAP kinase-3 can be activated by ERK, p38 MAPK, and JNK/SAPK (Ludwig *et al.*, 1996; McLaughlin *et al.*, 1996) and is thus an example of a substrate targeted by all three pathways. Both MAPKAP kinase-2 and MAPKAP kinase-3 are activated by cellular stresses and inflammatory cytokines, and both enzymes phosphorylate heat shock protein 27 (hsp27) (Engel *et al.*, 1995b, McLaughlin *et al.*, 1996; Clifton *et al.*, 1996). hsp27 behaves as an actin capping protein, thus MAPKAP kinases 2 and 3 are potential regulators of cytoskeletal assembly.

Mnk1 and 2 were identified in screens for ERK interacting proteins (Waskiewicz *et al.*, 1997; Fukunaga and Hunter, 1997). Both enzymes can be directly activated by ERK or p38 MAPK phosphorylation. Once activated, Mnk1 and 2 phosphorylate eukaryotic initiation factor 4E (Waskiewicz *et al.*, 1997) suggesting a mechanism for translational control by these two MAPK pathways.

B. Nuclear Transcription Factors

MAP kinases phosphorylate several proteins belonging to the Ets family of helix-turn-helix transcription factors. The p62 ternary complex factor (p62^{TCF}/Elk-1) regulates serum response element (SRE) transcription through its interaction with serum response factor (SRF) (Shaw *et al.*, 1989; Graham and Gilman, 1991). Transactivation is potentiated by phosphorylation of p62^{TCF}/Elk-1 by ERK, JNK/SAPK, or p38 MAPK (Marais *et al.*, 1993; Janknecht *et al.*, 1993; Gille *et al.*, 1992, 1995a,b; Cavigelli *et al.*, 1995; Whitmarsh *et al.*, 1995; Price *et al.*, 1996). Ternary complex factors Sap1 and Net/Erp/Sap2, which also interact with SRF and SRF, are substrates for ERK and p38 MAPK; Sap1 is also a substrate for JNK/SAPK (Price *et al.*, 1995, 1996; Janknecht *et al.*, 1995). Transactivators ERM/ER81 and most likely Ets1 and Ets2 are phosphorylated by ERK (Janknecht, 1996; Janknecht *et al.*, 1996; Conrad *et al.*, 1994). Ets family members that function as transrepressors are also directly regulated through phosphorylation by ERK. An Ets2 repressor factor (ERF), similar to p62^{TCF}/Elk-1 in its DNA-binding domain, is phosphorylated and inhibited in response to v-Raf in mammalian cells (Sgouras *et al.*, 1995). *In vitro*, ERF is phosphorylated by both ERK2 and cdc2 and is most likely regulated by phosphorylation at Thr_{526}, as substitution of this residue with Glu leads to loss of repression. Genetic screens for downstream transcriptional targets of ERK in *C. elegans* and *Drosophila* have so far identified mainly Ets family members as physiological substrates controlling developmental pathways (see Section V,B).

MAP kinases also target bZIP transcription factors for phosphorylation and activation. Both JNK/SAPK and p38 MAPK phosphorylate activating transcription factors ATFa and ATF2 (Gupta *et al.*, 1995, 1996; Bocco *et al.*, 1996), members of the ATF/CREB family that heterodimerize with c-Jun, NF-κB, and CREB. Phosphorylation enhances ATF2 transcriptional activity, whereas mutation of phosphorylation sites or expression of dominant negative mutant JNK/SAPK inhibits transcription (Gupta *et al.*, 1995). Dominant negative ERK mutants block transactivation by AP-1, showing that ERK activation is necessary for AP-1 transcriptional function (Frost *et al.*, 1994). Although ERK was originally found to phosphorylate c-Jun at Ser_{63}

and Ser_{73} within its activation domain (Pulverer *et al.*, 1991), later studies showed that ERK also phosphorylates residues at the C terminus of c-Jun, which may lead to negative regulation through inhibition of DNA binding (Alvarez *et al.*, 1991; Chou *et al.*, 1992; Minden *et al.*, 1994b). JNK/SAPK is the more likely physiological c-Jun kinase in mammalian systems because it forms a tight complex with c-Jun and phosphorylates it more efficiently (Hibi *et al.*, 1993; Minden *et al.*, 1994b; Dérijard *et al.*, 1994). In agreement, *in vivo* studies show a stronger correlation between c-Jun phosphorylation and JNK/SAPK activation than ERK activation (Smeal *et al.*, 1994; Westwick *et al.*, 1994). However, it is still possible that ERK activates AP-1 through direct phosphorylation of c-Jun under some conditions. For example, regulation of photoreceptor differentiation in *Drosophila* involves c-Jun phosphorylation at Ser_{82} and Ser_{92}, dependent on the activity of Rolled (Treier *et al.*, 1995; Peverali *et al.*, 1996). *Drosophila* JNK/SAPK is not required for photoreceptor differentiation, indicating that ERK is the physiological regulator of c-Jun in this system.

In vitro, Fos, Fra1, and Fra2 are potential targets for direct phosphorylation by ERK, as well as MAPKAP kinase-1, and sites phosphorylated on these transcription factors *in vitro* are similar to those observed *in vivo* (Chen *et al.*, 1993; Gruda *et al.*, 1994). In intact cells, phosphorylation of Fos in response to ERK stimulation leads to enhanced Fos protein stability (Okazaki and Sagata, 1995a). A serum stimulated Fos kinase (ERK) distinct from ERK was shown to activate AP-1 transactivation and may represent an equally important regulator of Fos (Deng and Karin, 1994).

c-Myc is phosphorylated by ERK at Ser_{62}, the primary site for phosphorylation *in vivo* (Alvarez *et al.*, 1991; Davis, 1993). This residue may be important for transcriptional regulation (Seth *et al.*, 1992), although conclusive evidence for transactivation through ERK is not available. In cell extracts, ERK binds tightly to the N terminus of c-Myc (Gupta and Davis, 1994), an interaction that is disrupted by Myc phosphorylation, suggesting functional interactions between enzyme and substrate. Another member of the Myc family, the N-Myc repressor, is phosphorylated by ERK at Ser_{51}. This phosphorylation event may be required for N-Myc function because mutation of Ser_{51} results in loss of repression (Manabe *et al.*, 1996).

ERK is also involved in steroid hormone receptor signaling, as demonstrated by its regulation of receptors for estrogen and peroxisome proliferator-activated receptor-γ (PPAR-γ). Estradiol activates ERK, most likely mediated through tyrosine phosphorylation of Shc (Migliaccio *et al.*, 1996). Transactivation of estrogen-response element/luciferase reporters by EGF is dependent on Ras and MKK and can be inhibited by MAP kinase phosphatase-1 expression, suggesting that transcriptional activity is mediated through ERK (Kato *et al.*, 1995; Bunone *et al.*, 1996). Correspondingly, estrogen receptor is phosphorylated by ERK at Ser_{118} *in vitro*; this residue is

necessary for optimal transcription because its mutation leads to decreased response. *In vivo*, however, Ser_{118} can be phosphorylated in the absence of estradiol, suggesting that ERK either mediates steroid-independent transactivation or coregulates steroid-dependent activation (Arnold *et al.*, 1995; Bunone *et al.*, 1996). The analogous residue (Ser_{112}) on the related PPAR-γ, a transcriptional regulator controlling adipocyte differentiation, is phosphorylated in response to EGF, phorbol esters, serum, or insulin (Hu *et al.*, 1996). Phosphorylation at Ser_{112} by ERK blocks transcriptional activity of PPAR-γ, providing a means by which growth factors may block adipogenesis through MKK/ERK signaling.

C. Signaling Components

Cytosolic substrates for MAP kinases include signaling components that may be involved in feedback regulation or cross-regulation of other pathways. A likely target for feedback inhibition by ERK is the Ras guanine nucleotide exchange factor, Sos1. *In vitro*, Sos1 is highly phosphorylated by ERK, leading to a characteristic retardation in gel mobility that is also observed in factor-stimulated cells (Chemiack *et al.*, 1994; Buday *et al.*, 1995). Peptide mapping studies have identified at least five residues on hSos1 that are phosphorylated by ERK, four of which are phosphorylated *in vivo* following growth factor stimulation (Corbalan Garcia *et al.*, 1996). Interestingly, not all of these sites conform to the consensus Ser/Thr–Pro recognition motif for the MAP kinases (Table II), suggesting some deviation in ERK substrate specificity. Disruption of the Sos1–Grb2 complex is sensitive to PD98059, indicating a functional role for MKK1 in Sos regulation (Dong *et al.*, 1996; Holt *et al.*, 1996). Part of this effect is insensitive to MKP-1 and may thus represent a unique example of ERK-independent MKK1 signaling. Phosphorylation of Sos1 following cell stimulation correlates with the destabilization of interactions among Sos1-Shc, Sos1-EGFR, and Sos1-Grb2 (Rozakis-Adcock *et al.*, 1995), thus interfering with Ras/Raf activation. Thus, through this mechanism, ERK may serve as its own negative regulator. Negative feedback regulation by ERK has also been proposed to occur through ERK phosphorylation of EGF receptor at Thr_{669} (Morrison *et al.*, 1996). However, the effect of phosphorylation at this site is ambiguous, as its mutation to Cys had no effect on receptor autophosphorylation or down-regulation of EGF signaling.

MAP kinases also phosphorylate enzymes involved in eicosanoid biosynthetic pathways. Cytosolic phospholipase A_2 (cPLA$_2$) is the rate-limiting enzyme in pathways of agonist-stimulated arachidonic acid release. *In vitro*, ERK phosphorylates cPLA$_2$ on Ser_{505}, resulting in elevated activity (Lin *et al.*, 1993; Nemenoff *et al.*, 1993; Gordon *et al.*, 1996). Temporal correla-

tions between the agonist stimulation of ERK and Ser_{505} phosphorylation versus the activation of $cPLA_2$ and its translocation to the plasma membrane support the importance of ERK as a physiological regulator of $cPLA_2$ (Durstin *et al.*, 1994; Sa *et al.*, 1995). Furthermore, in Madin–Darby canine kidney cells, activation of arachidonic acid release in response to stimulation of α_1 adrenergic receptors by epinephrine is inhibited by PD98059 (Xing and Insel, 1996). However, $cPLA_2$ can be regulated by other kinase cascades as well, depending on the cell type. For example, thrombin stimulation of $cPLA_2$ in platelets is insensitive to PD98059, but is completely blocked by SB203580, implicating a pathway mediated by p38 MAPK (Kramer *et al.*, 1996). Another potential target in eicosanoid signaling is cyclooxygenase-2 (COX-2) which is transcriptionally upregulated in response to all three MAPK pathways (A. Sorokin, personal communication). In addition, membrane translocation and activation of 5-lipoxygenase in response to the Ca^{2+} ionophore have been shown to be inhibited by PD98059 (Lepley and Fitzpatrick, 1996), implicating ERK in the regulation of leukotriene synthesis.

D. Cytoskeletal Proteins

Earlier studies demonstrated that addition of active ERK to cell-free extracts of interphase *Xenopus* oocytes resulted in microtubule polymerization resembling an M phase array (Gotoh *et al.*, 1991), suggesting a role of MAPK in meiotic spindle formation. Association of ERK with microtubule networks, determined by indirect immunofluorescence in mammalian cells has also been reported (Reszka *et al.*, 1995), although this is may be due to high levels of ERK expression in cells.

Several cytoskeletal substrates for MAP kinases are implicated in neuronal function. ERK was first described as a protein kinase that phosphorylates microtubule-associated proteins 1 and 2 (Hoshi *et al.*, 1988; Ray and Sturgill, 1987), although the physiological significance of these phosphorylation events has not been resolved. The microtubule-binding protein, Tau, is highly phosphorylated by ERK, as well as by other proline-directed (GSK-3, cdk5) and nonproline-directed (cAMP dependent kinase, protein kinase C, casein kinase I, CaM kinase II) protein kinases (Hosoi *et al.*, 1995; Singh *et al.*, 1996). ERK phosphorylation of Tau resembles an abnormal phosphorylation pattern associated with Alzheimer's disease and inhibits microtubule–Tau interactions (Drewes *et al.*, 1992). However, cotransfection of Tau with Raf-1 in COS cells does not result in Tau phosphorylation (Latimer *et al.*, 1995), suggesting that ERKs may not be as important as other proline-directed kinases. The neuronal intermediate filament, neurofilament-H, is phosphorylated by JNK/SAPK at repeated Lys–Ser(P)–Pro–X–Glu motifs within the C-terminal tail domain (Giasson *et al.*, 1996). Hyperphosphorylation of neurofilament H occurs in response to stress activation of JNK/SAPK in PC12 cells and dorsal root ganglia and is a characteristic feature of several neurodegenerative diseases. Synapsin I, an actin-binding protein involved in cross-linking synaptic vesicles with cytoskeleton, is phosphorylated concomitantly with ERK activation in response to neurotrophins (Matsubara *et al.*, 1996; Jovanovic *et al.*, 1996). Phosphorylation by ERK at Ser_{62}, Ser_{67}, and Ser_{551} of synapsin I reduces its F-actin bundling activity *in vitro*, whereas other proline-directed kinases had no effect.

V. RESPONSES TO MAPK PATHWAYS: GROWTH AND DIFFERENTIATION

A. Regulation of Cell Growth and Transformation

The rapid activation of Raf-1, MKK1/2, and ERK1/2 observed in response to mitogen stimulation implies that this pathway is necessary for cell cycle progression through G1. This is supported by evidence showing elevated DNA synthesis or cell growth under conditions of ERK activation such as expression of constitutively active mutant MKK1 (Brunet *et al.*, 1994; Seger *et al.*, 1994). Correspondingly, mitogen-induced DNA synthesis is blocked by antisense RNA-mediated ERK downregulation (Pagès *et al.*, 1993), dominant negative ERK mutants (Frost *et al.*, 1994; Troppmair *et al.*, 1994), or treatment of cells with the MKK inhibitor, PD98059 (Dudley *et al.*, 1995; Seufferlein *et al.*, 1996). In many cells, ERK activation occurs within minutes following factor treatment and declines after 1–2 hr. However, the kinetics can be variable under different conditions, raising the possibility that different responses may depend on the time frame of activation. For example, responses of CCL39 fibroblasts to thrombin, which is mitogenic, vs thrombin proteolytic peptide, which is nonmitogenic, respectively, occur with sustained vs transient kinetics of ERK activation (Vouret-Craviari *et al.*, 1993), suggesting that persistent activation of ERK is required for cells to pass G1. Likely targets for regulation by ERK during G1 are immediate early genes such as Fos and Egr-1, which can be induced through $p62^{TCF}$/Elk-1 phosphorylation and activation of serum response factor promoter elements (Kortenjann *et al.*, 1994; Hipskind *et al.*, 1994a,b; Beno *et al.*, 1995). Cyclin D1 is transcriptionally induced by MKK/ERK, and provides a link between the ERK pathway and S-phase entry through regulation of cyclin/cdk activity (Lavoie *et al.*, 1996). Phosphorylation of eIF4E by Mnk may also promote translation of key proteins needed for S phase entry (Waskiewicz *et al.*, 1997).

Although the best documentation of Raf/MKK/ERK activation occurs in

early G1, a few reports exist demonstrating Raf and ERK activation during mitosis in synchronized mammalian cells (Laird *et al.*, 1995; Edelmann *et al.*, 1996). Antibodies that specifically stain activated ERK show colocalization of ERK with spindle poles and condensed chromatin in mitotic cells (P. Shapiro and N. Ahn, unpublished results). In oocytes, ERK associates with microtubule organizing centers and is involved in meiotic spindle formation (Verlhac *et al.*, 1993). Furthermore, activation of ERK by v-Mos or constitutively active MKK1 leads to partial chromosome condensation and formation of novel germinal vesicle microtubule arrays (Choi *et al.*, 1996b). These data suggest a role for ERK in microtubule rearrangements during M phase.

Transfection of 3T3 cells with constitutively active mutant MKK1 and MKK2 (Mansour *et al.*, 1994a, 1996b; Brunet *et al.*, 1994b; Cowley *et al.*, 1994) demonstrated that MKK/ERK induces hallmarks of cellular transformation, such as high saturation density, anchorage independent growth, cell rounding, and solid tumor formation in mice. In addition, cell transformation induced by *v-ras* or *v-mos* can be blocked by dominant interfering mutants of MKK1 or ERK 1 or 2 (Troppmair *et al.*, 1994; Okazaki and Sagata, 1995b). Thus, sustained ERK activation most likely accounts for many of the cellular responses observed with oncogenic Ras, Raf-1, and Src. In somatic cells, elevated expression of v-Mos leads to irreversible growth arrest, apoptosis, and increased ploidy, effects that are blocked by p53 (Fukasawa *et al.*, 1995, 1997). This has led to the suggestion that constitutive MKK/ERK activation in the absence of p53 may enhance chromosome instability during cell transformation.

Although transformation is clearly dependent on Raf/MKK/ERK activation, the degree of tumorigenicity observed with activated forms of Raf or MKK1/2 is low compared to that observed with oncogenic *ras*. Interestingly, foci formation in NIH3T3 cells induced by *v-raf* can be synergistically enhanced to levels observed with *v-ras* by coexpression of GTPase deficient mutants of Rac1 or RhoA (Qiu *et al.*, 1995; Khosravi-Far *et al.*, 1995). Furthermore, dominant negative mutants of Rac or Rho blocked transformation in response to *v-ras*. Similar effects are observed with PI3'K, acting upstream of Rac (Rodriguez-Viciana *et al.*, 1997). Thus, activation of MKK/ERK is necessary but not sufficient to account for effects of Ras transformation, which instead requires the coordinated activation of other Ras effectors to control cytoskeletal reorganization.

B. Regulation of Cell Differentiation and Development

In contrast to the role of MKK1/2 and ERK1/2 in regulating cell growth, MAPK pathways also play an important role in regulating embryonic development and cell differentiation. This is best demonstrated in whole animal studies, where activation of MKK and ERK is essential for vulval development in *C. elegans*, photoreceptor cell specification and anterior–posterior body patterning in *Drosophila*, mesoderm induction during *Xenopus laevis* embryo development, and positive T-cell selection. In addition, MAPK cascades have been shown to regulate the differentiation of mammalian cell lines that undergo processes of commitment along neuronal, blood cell, or fat cell lineages. Such examples illustrate the ability of different cells to utilize identical pathways to control seemingly opposite responses of cell proliferation vs cell growth arrest and expression of lineage-specific genes.

1. WORM DEVELOPMENT

An ERK module is central to the process of vulval development in the *C. elegans* hermaphrodite. During vulval formation, a set of vulval precursor cells (VPCs) receive an inductive signal from the gonadal anchor cell (AC), directing those VPCs nearest the AC to adopt the vulval fate (1° or 2°). The pathways determining vulval fate have been analyzed genetically utilizing Vulvaless (Vul) and Multivulva (Muv) mutants (reviewed by Sundaram and Han, 1996). Characterization of these mutants has revealed a Ras-regulated MKK/ERK pathway analogous to that found in mammalian cells. The inductive signal is encoded by the *lin-3* gene, which is homologous to mammalian EGF (Hill and Sternberg, 1992). This signal is likely received at the VPC through the *let-23* receptor tyrosine kinase (RTK), a member of the EGF receptor subfamily (Aroian *et al.*, 1990, 1994). High dosages of *lin-3* result in 1° VPCs, whereas lower doses induce 2° VPCs, indicating that VPC fate is modulated by the strength of signaling through the pathway (Katz *et al.*, 1995). LET-23 appears to be localized to VPC cell junctions by LIN-2 and LIN-7, membrane-associated guanylate kinase (MAGUK)-like proteins (Hoskins *et al.*, 1996; Simske *et al.*, 1996). This localization may serve to position LET-23 toward the AC, the presumptive source of LET-3. SEM-5 is an adaptor protein homologous to mammalian Grb2 (Clark *et al.*, 1992), presumably binds activated LET-23 (Stern *et al.*, 1993), and associates with an as yet unidentified guanine-nucleotide exchange factor. Further downstream is the *let-60* gene, which encodes the *C. elegans* Ras protein (Han and Sternberg, 1990; Beitel *et al.*, 1990). LET-60 mediates the activation of the LIN-45 Raf homolog (Han *et al.*, 1993), which in turn activates a downstream MKK referred to as MEK-2 (Wu *et al.*, 1995; Kornfeld *et al.*, 1995a). MEK-2 functions upstream of the ERK homolog SUR-1/MPK-1 (Wu and Han, 1994; Lackner *et al.*, 1994). Downstream of SUR-1/MPK-1 are putative transcription factors, LIN-1 and LIN-31 (Miller *et al.*, 1993; Beitel *et al.*, 1995), whose corresponding promoter elements have yet to be identified. *lin-1* is an Ets family member, containing multiple ERK consensus phosphorylation sites, which negatively regulates vulval development, and *lin-31* is re-

lated to the HNF-3/*fork head* family of DNA-binding transcription factors. A reasonable model analogous to the behavior of mammalian ERK is that these transcription factors may be direct substrates for SUR-1/MPK-1 phosphorylation, activated through *let-60* signaling. A novel Raf-related kinase, KSR, has been identified genetically as an enhancer of *let-60* ras signaling in the vulval development system (Sundaram and Han, 1995; Kornfeld *et al.*, 1995b). KSR appears to function either downstream or in parallel to *let-60*. In support of the genetics, biochemical analysis of human KSR indicates that this component enhances signaling through accelerating MKK and ERK activation (Therrien *et al.*, 1996).

2 FRUIT FLY DEVELOPMENT

a The ERK Pathway in Eye Development

A variety of developmental processes in *Drosophila* are regulated by receptor tyrosine kinases. The *torso* receptor tyrosine kinase organizes the formation of the anterior and posterior regions of the embryo, whereas the *Drosophila* EGF receptor (DER) executes multiple developmental functions, including establishment of dorsal–ventral polarity of the egg (Sprenger *et al.*, 1989; Price *et al.*, 1989). Similarly, cell fate determination of the R7 photoreceptor cell in eye development is regulated by the *sevenless* RTK (Sev) (Hafen *et al.*, 1987). These receptor tyrosine kinases signal through common ERK pathway component genes, although they are differentially expressed and activated by distinct ligands (reviewed by Perrimon, 1994). The *sevenless* eye development pathway is well characterized and will be the focus of this discussion.

The *Drosophila* compound eye is composed of approximately 800 ommatidia (unit eyes), each of which contain eight photoreceptor cells (R1–R8). Photoreceptor differentiation occurs in an ordered sequence, first generating R8, followed by R2/R5 and R3/R4, R1 and R6, and finally R7 (reviewed by Yamamoto, 1994). Differentiation of the R7 photoreceptor, studied extensively using both genetic and molecular approaches, has established the ERK pathway as the mediator of the developmental signal. Signaling is initiated at the R7/R8 cell interface, where the *sevenless* receptor tyrosine kinase of R7 interacts with its ligand, bride of sevenless (Boss), a transmembrane protein expressed on the surface of the R8 photoreceptor (Krämer *et al.*, 1991). Upon dimerization and tyrosine autophosphorylation of Sev, the SH2-SH3 adaptor protein Drk(Grb2) binds Sev and recruits *Drosophila* Sos (Olivier *et al.*, 1993; Simon *et al.*, 1993), resulting in the activation of Ras1 (Simon *et al.*, 1991; Bonfini *et al.*, 1992). Ras1 activation is attenuated by the Ras GTPase, Gap1 (Gaul *et al.*, 1992). Another potential adaptor protein, daughter of sevenless (Dos), functions upstream of Ras1. Dos contains an N-terminal pleckstrin homology domain and is a substrate of the PTPase, corkscrew (Csw) (Raabe *et al.*, 1996; Herbst *et al.*, 1996). Although the function of *dos* is unclear, genetic relationships and biochemical data are consistent with a model in which dephosphorylation of Dos by Csw activates the signaling pathway downstream of Sev, leading to Ras1 activation. Following Ras1, *Drosophila* Raf-1 activates the *Drosophila* MKK1, D-Mek/Dsor1 kinase (Dickson *et al.*, 1992; Tsuda *et al.*, 1993; Hsu and Perrimon, 1994; Lu *et al.*, 1994). The *Drosophila* ERK homolog, Rolled, is then activated by D-Mek (Brunner *et al.*, 1994b; Biggs *et al.*, 1994). As discovered in *C. elegans*, KSR was identified in genetic screens as an enhancer of signaling, functioning downstream or in parallel to Ras1 (Therrien *et al.*, 1995). Interestingly, the protein phosphatase PP2A has appeared in genetic screens, both positively and negatively regulating the pathway downstream of Ras1; however, substrates for *Drosophila* PP2A are unknown (Wassarman *et al.*, 1996b). Thus, the fundamental ERK signaling pathway is conserved between invertebrates and mammals.

Several transcription factors regulated by the sevenless signaling cascade have been identified as Ets family members. *yan* and *pointed* (pnt) have negative and positive regulatory roles, respectively, in R7 photoreceptor differentiation (Lai and Rubin, 1992; O'Neill *et al.*, 1994; Brunner *et al.*, 1994a). The repressor activity of *yan* is abrogated on activation of the sevenless signaling pathway. The yan protein has multiple ERK consensus phosphorylation sites, and ERK phosphorylation of Yan appears to decrease its stability and alter its subcellular localization (Rebay and Rubin, 1995). The *pointed* gene encodes two related proteins, pntP1 and pntP2, which are products of alternative splicing. pntP1 is a constitutively active transcription factor, whereas pntP2 is transcriptionally active only when phosphorylated at its single ERK phosphorylation site (O'Neill *et al.*, 1994; Brunner *et al.*, 1994a). Thus, *yan* and *pointed* have opposing transcriptional activities and are differentially regulated by activation of the *sevenless* signaling pathway. *Drosophila* Jun (DJun) is also required for Ras-induced R7 photoreceptor differentiation (Bohmann *et al.*, 1994). DJun and Pointed synergistically activate transcription from an AP-1/Ets promoter element, indicating functional cooperation between these two transcription factors (Treier *et al.*, 1995). Further, *yan* antagonizes the enhanced Pointed/DJun transcriptional effect. DJun is a MAPK substrate, and its phosphorylation correlates with photoreceptor differentiation (Peverali *et al.*, 1996). Downstream target genes upregulated by signaling through the *sevenless* pathway include *phyllopod* and *seven in absentia* (sina), which encode nuclear proteins (Carthew and Rubin, 1990; Dickson *et al.*, 1995; Chang *et al.*, 1995). The *prospero* gene, a putative transcription factor involved in nervous system differentiation, is also upregulated in response to *sevenless* activation, and this induction appears to be mediated by *yan* and *pointed* (Doe *et al.*, 1991; Kauffmann *et al.*, 1996). Intriguingly, Phyllopod is also able to upregulate

prospero in coordination with Sina. Phyllopod forms a complex with Sina both *in vitro* and *in vivo*, and it is likely that the Phyllopod/Sina complex has transcriptional activity.

b. The JNK/SAPK Pathway in Epithelial Cell Migration

A second *Drosophila* MAPK pathway is important for dorsal closure and the insect immune response, representing a unique example of the role of the JNK/SAPK pathway in development. Dorsal closure occurs at midembryogenesis and entails the dorsalward spreading of epidermal cells over the amnioserosa membrane until fusion occurs at the dorsal midline (Young *et al.*, 1993). Interestingly, this developmental process is regulated by the JNK/SAPK pathway (see Section II,B). The loss of function *hemipterous* gene is defective in dorsal closure and encodes a *Drosophila* JNK kinase (Hep) that is homologous to mammalian MKK3 and MKK4 (Glise *et al.*, 1995). Hep is able to phosphorylate and activate a JNK/SAPK homolog, DJNK, encoded by the *basket* gene (Sluss *et al.*, 1996). Embryos lacking DJNK or that express dominant negative *Drosophila* cdc42, an upstream GTPase, are also deficient in dorsal closure (Sluss *et al.*, 1996; Riesgo-Escovar *et al.*, 1996). Thus, JNK/SAPK pathway components appear to be conserved between mammals and flies. A *Drosophila* MEKK, Pk92B, has been cloned (Wassarman *et al.*, 1996a). Although its role in development has not been reported, it is reasonable to expect a function upstream of Hep. In addition, DJNK is activated on exposure of cultured *Drosophila* cells to endotoxic lipopolysaccharide. DJNK activation coincides with a marked induction of antibacterial immune response genes, indicating that the JNK/SAPK pathway also mediates an immune response (Sluss *et al.*, 1996). Both developmental and immune responses are likely to be dependent on activation of the DJun transcription factor. Like the ERK homolog, *rolled*, DJNK efficiently phosphorylates DJun, yet the two pathways appear to be distinct as DJNK is not required in eye development (Sluss *et al.*, 1996; Riesgo-Escovar *et al.*, 1996). Interestingly, the Hep–DJNK pathway upregulates the expression of the *puckered* gene, which encodes a MAPK phosphatase (Riesgo-Escovar *et al.*, 1996). Hence, regulation through a negative feedback loop is another conserved feature of the pathway.

3. MESODERM INDUCTION IN *Xenopus* EMBRYOS

A role for MKK/ERK in vertebrate development has been documented in the early process of mesoderm induction. MKK and ERK act as cytostatic factors following the fertilization of *Xenopus* oocytes when their activities drop (Haccard *et al.*, 1993; Kosako *et al.*, 1994); however, their activities appear again following the blastula stage (MacNicol *et al.*, 1995). In *Xenopus* embryos, signals derived from the vegetal hemisphere, including activin and basic fibroblast growth factor (bFGF), diffuse into the animal pole, leading to changes in gene expression and morphogenesis. Treatment of explanted animal poles with various factors show that signaling through bFGF is a key regulator of mesodermal induction, synergistic with activin signaling (reviewed by Gotoh and Nishida, 1996). The importance of Ras and Raf was indicated by inhibition of mesoderm induction on microinjection of dominant negative mutants of these enzymes into animal pole explants treated with bFGF (Whitman and Melton, 1992; MacNicol *et al.*, 1993; Gotoh *et al.*, 1995b). bFGF, but not activin, leads to the stimulation of ERK (Graves *et al.*, 1994), and the involvement of MKK/ERK in bFGF signaling was confirmed by the induction of mesoderm on injection of constitutively active MKK, ERK (sevenmaker), or STE11 mutants into explants (Labonne and Whitman, 1994; Gotoh *et al.*, 1995; Umbhauer *et al.*, 1995). The resulting mesodermal tissue was capable of undergoing further morphogenesis and cardiac actin expression by the dorsalizing factor, noggin. Likewise, the bFGF-induced mesodermal development was inhibited by microinjection of MAP kinase phosphatase-1. Thus, signaling through MKK/ERK appears to be sufficient to mimic the effects of bFGF and most likely targets early regulators of mesoderm development, such as the *Xenopus* Brachyury (Xbra) immediate early gene.

4. NEURONAL DIFFERENTIATION

One approach to understanding the processes of differentiation at a mechanistic level is by manipulating cell lines that differentiate in culture. The rat pheochromocytoma (PC12) line is a widely used model for neuronal differentiation, in which nerve growth factor (NGF) or bFGF cause cell cycle arrest in G1, cytoskeletal rearrangements leading to neurite outgrowth, and expression of neuronal markers. Inhibition of MKK1/2 with PD98059 suppresses NGF-induced neuronal differentiation (Pang *et al.*, 1995), and expression or microinjection of constitutively active MKK1 induces neurite outgrowth in the absence of NGF (Cowley *et al.*, 1994; Fukuda *et al.*, 1995). The inhibition of cell proliferation in response to NGF is mediated through the induction of p21(Cip1/Waf1) and suppression of cyclin/cdk activity (Pumiglia and Decker, 1997). This is blocked by PD98059, indicating that MKK/ERK induces G1 cell cycle arrest through control of p21(Cip1/Waf1) transcription. Under certain conditions, positive correlations between ERK activation and differentiation are not observed. For example, activation of ERK by mutant NGF or PDGF receptors does not necessarily lead to cell differentiation (Peng *et al.*, 1995; Vaillancourt *et al.*, 1995), and induction of cell differentiation by bone morphogenetic protein or activin A does not require ERK activation (Iwasaki *et al.*, 1996). Therefore, the sufficiency of MKK/ERK activation in regulating PC12 cell differentiation is debatable.

Nevertheless, MKK/ERK appears to play an essential role in controlling neuronal differentiation in response to NGF or bFGF signaling.

An interesting feature of PC12 cells is that ERK is activated in response to both proliferative and differentiative stimuli. Several laboratories have reported short term activation of ERK, which is downregulated within a few hours following treatment of cells with proliferation factors vs prolonged activation of ERK on treatment with differentiation factors (Gotoh *et al.*, 1990a; Qiu and Green, 1992; Traverse *et al.*, 1992). This prolonged activation can last up to 10 days and appears to be necessary to maintain the differentiated state and cell survival. The observed variation in kinetics of ERK activation has suggested that sustained activation of ERK may be necessary to trigger a late event necessary for differentiation, but not proliferation, of PC12 cells. A similar model has been proposed to explain proliferative responses in CCL39 cells (Vouret-Craviari *et al.*, 1993; see Cell Growth above). In both cases, nuclear translocation of ERK occurs only in response to prolonged activation (Traverse *et al.*, 1992; Vouret-Craviari *et al.*, 1993), suggesting that the late event responsible for the differentiation of PC12 cells or mitogenesis of CCL39 cells involves the phosphorylation of nuclear targets.

5. BLOOD CELL DIFFERENTIATION

MAPK activation occurs as an acute response in lymphoid cells to T-cell receptor or B-cell receptor stimulation (Whitehurst *et al.*, 1992; Izquierdo *et al.*, 1993; Tordai *et al.*, 1994; Nel *et al.*, 1995). In mature T cells that respond to TCR stimulation by transcription of interleukin-2, proliferation, or apoptosis, MAPKs are stimulated through a pathway involving coupling of the TCR–CD3 complex to Ras and Raf, mediated by Lck and ZAP70 tyrosine kinase interactions with TCR. ERK and JNK/SAPK activation appear to be involved in the transcriptional induction of interleukin-2, and TCR signaling is blocked by dominant negative mutant MEKK1 in these pathways (Faris *et al.*, 1996). Inhibition of IL-2 transcription also correlates with suppressed ERK and JNK stimulation following T-cell anergy produced in the absence of receptor costimulation (Li *et al.*, 1996).

Immature $CD4^-/CD8^-$ T cells respond to TCR stimulation by positive selection pathways, controlling T-cell differentiation into $CD4^+/CD8^+$ cells, or negative selection, controlling cell death. The involvement of MKK/ERK in TCR-regulated pathways was investigated by targeting a dominant negative mutant MKK1 for expression in T cells under control of the *lck* proximal promoter (Alberola-Ila *et al.*, 1995). Mice expressing this mutant showed lowered levels of $CD4^+8^-$ or $CD4^-8^+$ cells, indicating a requirement for MKK1 during maturation of double-positive to single-positive thymocytes. No effects on negative T-cell selection or TCR-induced proliferation or apoptosis were observed, suggesting an important role of alternative signaling pathways, such as those involving stress-activated kinases (Sen *et al.*, 1996). Results consistent with these studies were observed in fetal thymic tissue utilizing retroviral transfection of MKK1 into organ cultures (Crompton *et al.*, 1996). Both double-positive and single-positive T cells were increased following transfection of constitutively active MKK1 into tissue from TCRα-deficient mice, and transfection of dominant negative MKK1 blocked differentiation. No evidence for an effect of MKK1 on T-cell proliferation was observed, in agreement with the whole animal study.

A role for MKK/ERK in the differentiation of other blood cell types has also been examined by expression of constitutively active MKK mutants. A late event in blood cell differentiation is the commitment of nonlymphoid cells into megakaryocyte versus erythroid lineages. The human erythroleukemia cell lines, K562 and CMK, retain the capacity to differentiate into megakaryocytes in response to phorbol ester. In either cell type, expression of constitutive active MKK1 leads to inhibition of cell growth, induction of characteristic megakaryocytic morphology, including increased cell size and multinucleation, and cell surface expression of integrin $\alpha_{IIb}\beta_3$, an adhesion receptor specifically expressed in platelets (Whalen *et al.*, 1997; Vik *et al.*, 1997). In addition, the expression of globin genes in K562 cells is blocked by elevated MKK/ERK activity and is upregulated by the PD98059 inhibitor (Whalen *et al.*, 1997), thus MKK/ERK promotes megakaryocyte differentiation at the same time it suppresses erythroid differentiation. These results illustrate an example of how MKK/ERK regulates lineage commitment in pluripotent cells at the level of marker gene expression.

6. ADIPOCYTE DIFFERENTIATION

Adipocyte differentiation from fibroblasts involves a series of transcriptional events leading to growth arrest and induction of specific genes required for fat synthesis and degradation (reviewed by MacDougald and Lane, 1995). The 3T3-L1 cell line is a useful model that proliferates with a fibroblast morphology, but can be induced to differentiate into adipocytes by continuous exposure to insulin-like growth factor 1, glucocorticoid, fatty acids, and agents that elevate cAMP. Earlier studies showed induction of differentiation by expression of v-*ras* or v-*raf* in 3T3-L1 fibroblasts (Benito *et al.*, 1991; Porras *et al.*, 1994). The evidence implicating ERK in this process is conflicting, however. In one study, ERK antisense oligonucleotides, used to inhibit ERK activation in response to insulin, successfully blocked insulin induced differentiation of 3T3-L1 fibroblasts (Sale *et al.*, 1995). In another study, differentiation could be activated by v-*raf*, as scored by the accumulation of fat droplets and transcription of the aP2 lipid-binding protein, but activation of ERK or Rsk was not observed under these conditions,

suggesting that differentiation through Ras and Raf occurs through pathways separate from those involving MKK/ERK (Porras *et al.*, 1994). More recent studies have shown that the expression of constitutively active MKK1 inhibits insulin induced adipocyte differentiation, whereas the PD98059 MKK inhibitor suppresses ERK activation, with no effect on differentiation (Font de Mora *et al.*, 1997). It thus appears that the primary effect of MKK/ERK is to negatively regulate adipogenesis and that v *raf* may positively regulate differentiation through an alternative pathway.

Among the important tissue-specific regulators of adipogenesis are the transcriptional activators C/EBP/enhancer binding protein (C/EBPα, β, δ) and peroxisomal proliferator activated receptor γ2 (PPAR-γ); the latter mediates differentiation in response to thiazolidinediones and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (Wu, Z. *et al.*, 1996). PPAR-γ levels increase concomitantly with C/EBPα, and together they synergize in promoting differentiation (Lin and Lane, 1992; Tontonoz *et al.*, 1994; Brun *et al.*, 1996). The inhibition of adipocyte differentiation by MKK/ERK can be explained by the finding that ERK2 phosphorylates PPAR γ2, most likely at Ser_{112}. Mutation of Ser_{112} to Ala did not affect ligand induced transcription, but relieved repression of transcription in response to phorbol ester (Hu *et al.*, 1996), suggesting that ERK phosphorylation inhibits adipocyte differentiation by suppressing PPAR γ2 transactivation. The C/EBP family member, CHOP/Gadd153, inhibits adipocyte differentiation through heterodimerization with other C/EBP family members. In separate studies, phosphorylation of CHOP/Gadd153 by p38 MAPK was found to be necessary for a full inhibitory effect (Wang and Ron, 1996), providing a mechanism by which ERK and p38 MAPK cooperate to antagonize differentiation in this system.

VI. YEAST MAPK PATHWAYS

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have provided an important complement to metazoan systems in the dissection of MAPK pathways. Through the use of genetic and molecular approaches, five distinct MAPK pathways have been revealed in *S. cerevisiae* and three in *S. pombe*. There are striking similarities between yeast and metazoan MAPK signaling, especially at the level of the MAPK module, phosphatase regulation, and transcriptional control. However, the yeasts lack homologs of Raf and appear to signal exclusively through MEKK-like kinases. In addition, the yeasts have thus far been shown to use heterotrimeric G protein coupled seven transmembrane domain receptors and, in one case, a two component system, thus receptor tyrosine kinases appear to be utilized exclusively in multicellular systems. Nevertheless, yeast models have led to important insights into the dual function of small GTPases in cytoskeletal organization and signaling, as well as mechanisms of heterotrimeric G protein signaling to MAPK modules.

A. *Saccharomyces cerevisiae* (Budding Yeast)

1. PHEROMONE RESPONSE PATHWAY

Mating in *S. cerevisiae* occurs between haploid cells of opposite mating types (α or **a**), and is characterized by the induction of mating-specific genes, cell cycle arrest, and cell morphology changes. This process occurs through activation of the pheromone response pathway, which employs a MAPK module composed of STE11, STE7, and FUS3/KSS1 (Fig. 2). The pheromone response is initiated when α or a peptide mating pheromone binds the appropriate seven transmembrane domain cell surface receptor (STE2 or STE3, respectively), which activates an associated heterotrimeric G protein through guanine nucleotide exchange, releasing G_{α}-GTP from $G_{\beta\gamma}$. The $G_{\beta\gamma}$ dimer (STE4 and STE18) activates the downstream MAPK cascade through an incompletely understood mechanism, resulting in the activation of STE20 serine/threonine kinase. The small GTPase, CDC42, and its GTP exchange factor, CDC24, are required in the pheromone response pathway. CDC42 binds to and activates STE20 (Simon *et al.*, 1995; Zhao, Z. *et al.*, 1995), as observed with mammalian Cdc42 and PAK. Although STE20 is required for mating, this interaction functions to localize STE20 to sites of bud emergence (Peter *et al.*, 1996; Leberer *et al.*, 1997) and may not be a necessary event in MAPK signaling. Once activated, STE20 phosphorylates and presumably activates STE11 (MEKK) (Wu *et al.*, 1995), although it is not clear how phosphorylation leads to activation. In analogy to the mammalian kinase cascades, STE11 phosphorylates and activates STE7 (MKK) (Neiman and Herskowitz, 1994), which in turn phosphorylates and activates FUS3 and KSS1 (MAPKs) (Gartner *et al.*, 1992; Errede *et al.*, 1993; Ma *et al.*, 1995).

FUS3 has the dual task of controlling the transcription of genes involved in mating and inducing cell cycle arrest. STE12, a transcription factor, and FAR1, a cell cycle regulator, are both substrates for FUS3 (Elion *et al.*, 1993; Peter *et al.*, 1993). Phosphorylation of STE12 is thought to activate transcription of mating specific genes, such as FUS1and FAR1 (Trueheart *et al.*, 1987; Oehlen *et al.*, 1996), and phosphorylation of FAR1 arrests the cell cycle at G1 by associating with and inhibiting the cyclin-dependent protein kinase, CDC28-CLN2 (Peter *et al.*, 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994). Hence, as in mammalian systems, external signals received at the membrane are transduced to the nucleus via a MAPK cascade.

STE5 is a signaling molecule that appears to serve as a scaffold for other

components in the cascade (Choi *et al.*, 1994; Printen and Sprague, 1994; Marcus *et al.*, 1994). STE11, STE7, and FUS3/KSS1 all bind independently to different regions of STE5, thus enabling association into a multikinase complex. Only the active form of STE11 is present in the STE5 complex, suggesting a mechanism whereby STE5 binding facilitates STE11 activation. Indeed, the N terminus of STE11 (amino acids 1–435), which is required for STE5 interaction, also functions as a negative regulatory domain (Cairns *et al.*, 1992). STE5 complexes also bind hypophosphorylated STE7, suggesting that STE5 may recruit active STE11 to inactive STE7 and FUS3/KSS1. Activated STE7 and FUS3/KSS1 may then be released, allowing FUS3/KSS1 to phosphorylate downstream targets (Choi *et al.*, 1994). In absence of STE5 (*ste5Δ*), complexes among STE11, STE7, and FUS3/KSS1 are still able to form, suggesting that other mechanisms exist to account for the role of STE5 in mating. However, although STE11 and STE7 interact with FUS3/KSS1, STE11 associates only marginally with STE7 (Choi *et al.*, 1994; Printen and Sprague, 1994; Bardwell *et al.*, 1996). Hence, facilitation of multikinase complexes by STE5 is probably important for signal transmission.

Protein–protein interactions are implicated with other components that play a vital role in the pheromone response pathway. STE4 (G_β) binds to the N terminus of STE5, potentially mediating signaling from heterotrimeric G proteins to the MAPK module (Whiteway *et al.*, 1995). STE4 also binds the N terminus of the guanine nucleotide exchange factor CDC24 (Zhao, Z. *et al.*, 1995). This suggests a model in which the STE4–STE5 complex activates CDC42 and recruits STE20 (via STE4), bringing it in proximity to its substrate, STE11 (via STE5). The SH3 domain-containing BEM1 protein, involved in cell polarity, is required for efficient signaling in the pheromone response pathway. In a situation analogous to STE4, BEM1 directly binds CDC24 (Peterson *et al.*, 1994) and interacts with both STE20 and STE5, as well as actin (Leeuw *et al.*, 1995). In addition to binding the STE5 complex, BEM1 associates with FAR1 (Lyons *et al.*, 1996). Conceivably, BEM1 could serve to localize the MAPK module, including its upstream activating components as well as the downstream FAR1 substrate, at the shmoo tip via its association with the actin cytoskeleton. The involvement of BEM1/CDC24/CDC42 in the pheromone response pathway demonstrates that components necessary for cytoskeletal reorganization are also fundamental in signal transduction, reminiscent of Rac/Rho function in mammalian systems.

In addition to facilitation of signaling through complex formation, STE5 might prevent cross-talk between the pheromone response pathway and the other *S. cerevisiae* MAPK pathways. In support of this, a STE7 gain of function mutant can complement a mutation in the cell wall integrity pathway (*bck1Δ*) only when it is overproduced or when STE5 is missing (Yashar *et al.*, 1995).

Desensitization of the pheromone response pathway involves dephosphorylation and inactivation of FUS3. The dual specificity phosphatase MSG5 (Doi *et al.*, 1994) appears to regulate this process because loss of the MSG5 gene results in diminished desensitization. Thus, a phosphatase homologous to the VH1-like class of dual specificity phosphatases, which in humans includes MKP-1 and PAC1 (Table I), is involved in inactivating yeast MAPK. Mating pheromone treatment enhances MSG5 expression by fivefold within 30 min. This supports a negative feedback mechanism in which activation of FUS3 causes the transcriptional upregulation of MSG5, as is seen with mammalian MKP-1 and MKP-2 on serum stimulation (Brondello *et al.*, 1997).

2. INVASIVE/PSEUDOHYPHAL GROWTH

Invasive growth, characterized by filament formation and agar penetration, occurs in haploid yeast (α or a mating type) when nutritionally starved for nitrogen (Roberts and Fink, 1994). This pathway shares many components with the pheromone response pathway, including STE20 (PAK), STE11 (MEKK), STE7 (MKK), and STE12 (transcription factor). Invasiveness does not require pheromone receptor, G proteins (STE4, STE18), STE5, or FAR1, leaving the signal input component unknown. Deletion of both *FUS3* and *KSS1* does not impair invasive growth; however, loss of KSS1 alone shows impaired agar penetration, suggesting that KSS1 regulates the invasive response. Indeed, KSS1 in its inactive state negatively regulates invasive growth, yet on activation by STE7, KSS1 promotes invasiveness (Cook *et al.*, 1997). Interestingly, mating specific genes are not induced, despite the presence of STE12 in the invasive growth pathway (see below). Two homologous proteins, DIG1 and DIG2 (RST1 and RST2), have been identified that suppress invasive growth (Cook *et al.*, 1996; Tedford *et al.*, 1997). Both DIG1 and DIG2 coprecipitate with KSS1 and FUS3, indicating that they interact *in vivo*, and DIG1/2 bind STE12. DIG1 colocalizes with KSS1 in the nucleus, and both DIGs are phosphorylated by KSS1. These data suggest a model in which DIGs inhibit transcription from filamentous growth response elements (FREs), perhaps through binding of STE12. However, on pathway stimulation induced by starvation, KSS1 might phosphorylate DIG1/2, thereby relieving transcriptional repression and allowing invasive growth.

Pseudohyphal growth occurs in diploid (α/a) cells and, like invasive growth, entails the formation of agar-penetrating filamentous growth in response to nitrogen starvation (Gimeno *et al.*, 1992; Kron *et al.*, 1994). STE20, STE11, STE7, and STE12 are essential to pseudohyphal growth (Liu *et al.*, 1993); and, as is the case for the invasive pathway, KSS1 is the MAPK targeted by STE7 (Cook *et al.*, 1997). Upstream components have also been further characterized in this system. Constitutive activation of RAS2 (through

mutant RAS2^{Val19}) enhances pseudohyphal growth (Gimeno *et al.*, 1992). This may be mediated through CDC42, which has also been shown to regulate filamentous growth through binding of STE20, and functions downstream of RAS2 (Mösch *et al.*, 1996; Peter *et al.*, 1996; Leberer *et al.*, 1997). However, RAS2 does not function in the pheromone response pathway and thus mediates signaling from a different source into the same MAPK module. Similarly, the 14-3-3 homologs BMH1 and BMH2 are essential for pseudohyphal growth signaling, but not pheromone signaling (Roberts *et al.*, 1997). BMH1 and BMH2 may function in the pathway via interaction with STE20. Activation of the pseudohyphal growth pathway does not activate mating specific genes, analogous to the invasive response (Mösch *et al.*, 1996). Rather, STE12 and the transcription factor TEC1 cooperatively bind to TREs to promote transcription required for both invasive and pseudohyphal development (Gavrias *et al.*, 1996; Madhani and Fink, 1997). Other possible targets of the pseudohyphal growth MAPK module are MSS10 (a transcription factor) and MUC1 (homologous to mucin like membrane proteins), both of which are essential for pseudohyphal differentiation (Lambrechts *et al.*, 1996).

3. HIGH OSMOLARITY RESPONSE (HOG PATHWAY)

In *S. cerevisiae*, increases in external osmolarity are compensated for by increased glycerol synthesis and decreased glycerol permeability (Luyten *et al.*, 1995). This high osmolarity glycerol response is rare among eukaryotic signaling pathways in that it combines a two-component response regulatory system (reviewed by Appleby *et al.*, 1996) with a MAPK module composed of SSK2/SSK22 (redundant MEKKs), PBS2 (MKK), and HOG1(MAPK) (Fig. 2). Two distinct cell surface receptors, SLN1 and SHO1, act as osmosensors that regulate the HOG cascade.

SLN1 was initially identified in a screen for synthetic lethal mutations requiring the ubiquitin protein-degradation pathway (Ota and Varshavsky, 1993). Both PBS2 and HOG1 were identified as osmoregulation-defective mutants (Brewster *et al.*, 1993), and SSK2 and SSK22 were discovered in a screen for extragenic suppressor mutants of the lethal *SLN1* null mutation (Maeda *et al.*, 1995). Strikingly, SLN1 is a transmembrane protein with homology to bacterial two-component receptors, containing osmotic sensor, histidine kinase transmitter, and phosphate receiver domains. The remainder of this two-component system includes the response regulators YPD1 and SSK1 (Maeda *et al.*, 1994; Posas *et al.*, 1996), activating the HOG pathway at the level of SSK2 and SSK22 (Maeda *et al.*, 1995). Under conditions of low osmolarity, SLN1 autophosphorylates at His_{576} in its transmitter domain. The phosphoryl group is first transferred to Asp_{1144} in the SLN1 receiver domain, from which it is transferred to His_{64} on YPD1. Finally, the phosphoryl group is passed to Asp_{554} in the C-terminal receiver domain of SSK1 (Posas *et al.*, 1996). SSK1 is phosphorylated and inactive when the external osmolarity is low. Under conditions of high osmolarity, the SLN1 histidine kinase is inactivated and the resulting unphosphorylated SSK1 activates the downstream SSK2/SSK22, most likely by binding the N-terminal inhibitory domain of SSK2 or SSK22 and relieving autoinhibitory constraints (Maeda *et al.*, 1994). Upon activation, SSK2 or SSK22 phosphorylates and activates PBS2, which in turn phosphorylates and activates HOG1. Thus, histidine kinase activity negatively regulates HOG1, and *sln1Δ* mutant strains are lethal due to hyperactivation of the HOG pathway.

The second osmosensor, SHO1, is a putative transmembrane protein needed for viability in *sln1Δ* strains. SHO1 associates with PBS2 through the binding of its SH3 domain to a proline-rich domain in PBS2 (Maeda *et al.*, 1995). This interaction activates PBS2 through phosphorylation; however, neither SSK2 nor SSK22 is involved. Instead, SHO1 binding recruits PBS2 to a multikinase complex with STE11 and HOG1, although no crossregulation of HOG by pheromone is apparent (Posas and Saito, 1997).

Once activated, HOG1 completes the signaling response by inducing the transcription of genes necessary for maintaining osmotic balance. Among these are glycerol-3-phosphate dehydrogenase (GPD1) (Albertyn *et al.*, 1994) and glycerol-3-phosphatase (GPP2) (Norbeck *et al.*, 1996), both essential for glycerol synthesis. Other HOG1-regulated genes include a cytosolic catalase T, CTT1 (Schüller *et al.*, 1994), and a small heat shock protein, HSP12 (Varela *et al.*, 1995), both of which are under the control of stress response elements (STREs). The zinc finger transcription factors MSN2 and MSN4 are important regulators of STRE-dependent promoters and are required for activation of *CTT1* and *HSP12* genes (Schmitt and McEntee, 1996; Martínez-Pastor *et al.*, 1996). Strains deficient in *MSN2* and *MSN4* are less sensitive to high osmolarity than *hog1Δ* strains but are still viable; therefore, not only do these transcription factors function in HOG signaling, other factors are likely to be involved.

The HOG pathway can be downregulated by protein tyrosine phosphatases PTP2 (Ota and Varshavsky, 1992; Guan *et al.*, 1992; James *et al.*, 1992) and PTP3 (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997), as well as PTC1, PTC2, and PTC3, which are homologous to the mammalian serine/threonine phosphatase, PP2C (Maeda *et al.*, 1993; I. Ota, personal communication). PTPs 2 and 3 and PTCs 1–3 are able to suppress lethality in *sln1Δ* strains when overexpressed, indicating that these phosphatases suppress hyperactivation of the HOG pathway (Maeda *et al.*, 1994; I. Ota, personal communication). PTP2 and PTP3 target HOG1, whereas the substrate specificities of PTCs 1, 2, and 3 have yet to be determined in this system (Table I). It has been suggested that the PTCs regulate both PBS2 and HOG1 activity, although this has not been directly demonstrated. Both

PTP2 and PTP3 are transcriptionally induced in response to hyperosmolarity in a HOG1 dependent manner (Jacoby *et al.*, 1997), thus downregulation of the pathway can be controlled through a negative feedback mechanism.

4 CELL WALL INTEGRITY (PKC PATHWAY)

A MAPK cascade modulates part of a pathway regulating cell wall integrity. *S. cerevisiae* has a single PKC gene (PKC1) that is closely related to the mammalian PKC α, β, and γ isoforms (Levin *et al.*, 1990). Mutations that inactivate PKC1 result in cell lysis, caused by deficiencies in cell wall construction (Levin and Bartlett Heubusch, 1992; Paravicini *et al.*, 1992). Downstream of PKC1 is an MEKK (BCK1/SLK1), which, when mutated, also results in cell lysis (Lee and Levin, 1992; Costigan *et al.*, 1992). BCK1 has a PKC consensus phosphorylation site at Ser_{1134}, and PKC1 selectively phosphorylates BCK1 *in vitro* (Levin *et al.*, 1994), suggesting that PKC1 activates BCK1 through phosphorylation. In addition, two functionally redundant MKKs in this pathway (MKK1 and MKK2) suppress a BCK1 deletion mutant when overexpressed (Irie *et al.*, 1993). MKK1 and MKK2 are upstream of and activate a MAPK, MPK1/SLT2 (Torres *et al.*, 1991; Lee *et al.*, 1993), which, like FUS3, is negatively regulated by the dual specificity phosphatase MSG5 (Watanabe *et al.*, 1995). Although the PKC1 pathway does not appear to involve a STE5-like protein, MKK1 interacts with PKC1, whereas MKK1 and MKK2 bind both BCK1 and Mpk1 (Soler *et al.*, 1995; Paravicini and Friedli, 1996). Thus, the kinase components form a multienzyme complex through association with MKK1/2. Although each component in this pathway (BCK1, MKK1/2, and MPK1) yields a cell lysis defect when deleted, none is as severe as the loss of PKC1. This implies that the MAPK module functions only in one branch of a bifurcated pathway regulated by PKC1 (Errede and Levin, 1993).

Upstream regulation of PKC1 involves a number of stimulatory inputs. Tyrosine phosphorylation and activation of MPK1 increase in response to hypotonic shock (Davenport *et al.*, 1995) or mild heat shock (Kamada *et al.*, 1995). In addition, MPK1 is activated during periods of polarized cell growth (budding) in a CDC28-dependent manner (Mazzoni *et al.*, 1993; Zarzov *et al.*, 1996; Marini *et al.*, 1996). Each of these events disrupts the structural integrity of the plasma membrane, suggesting a mechanism whereby plasma membrane stretch activates mechanosensitive ion channels, resulting in an influx of Ca^{2+} (Kamada *et al.*, 1995).

Increased intracellular Ca^{2+} might activate PKC1 directly or indirectly through the stimulation of phospholipase C (PLC) and diacylglycerol synthesis. In the case of polarized cell growth, CDC28 upregulates diacylglycerol production, possibly through activation of a phosphatidylcholine-specific PLC (Marini *et al.*, 1996). Although PKC1 is not activated by phospholipids, Ca^{2+}, or DAG *in vitro*, it is conceivable that the proper conditions have not been found (Watanabe *et al.*, 1994). For example, the phospholipid dependent regulation of PKC1 is facilitated by RHO1-GTP, a homolog of mammalian RhoA, which binds PKC1 at its pseudosubstrate site and C1 domain (Nonaka *et al.*, 1995). When complexed with RHO1-GTP, PKC1 is strongly activated by phosphatidylserine (Kamada *et al.*, 1996), which serves as the exclusive cofactor in the RHO1/PKC1 complex, as neither Ca^{2+} nor DAG is stimulatory. A RHO1 loss of function mutation is complemented by overexpression of MPK1 or a gain of function MKK1, and RHO1 is required for MPK1 activation during heat shock (Kamada *et al.*, 1996). These results suggest that PKC1 is a target for RHO1 and that RHO1 dependent stimulation of PKC1 activates the downstream MAPK cascade (Nonaka *et al.*, 1995).

Other evidence suggests that PKC1 might be regulated by phosphoinositides. A yeast phosphatidylinositol 4-kinase (STT4) has been shown genetically to function upstream of PKC1 and BCK1 (Yoshida *et al.*, 1994). However, a strain bearing an STT4 deletion is incompletely suppressed by overexpression of PKC1 or a gain of function BCK1 mutant, implying that a second branch point likely exists between STT4 and PKC1.

Targets of the MAPK cascade in the PKC1 pathway are just beginning to be defined. Two high-mobility group (HMG)-like proteins (NHP6A and NHP6B) function downstream of MPK1, although they are not likely to be direct targets (Costigan *et al.*, 1994). A transcription factor (RLM1) with homology to the MADS box family is a probable target of MPK1 induction (Watanabe *et al.*, 1995). Five genes involved in cell wall biosynthesis are upregulated by MPK1, including those encoding enzymes needed in the synthesis of (1–3)-β-glucan, (1–6)-β-glucan, chitin, and N- and O-linked mannoproteins (Igual *et al.*, 1996). Upstream regulatory promoter sequences and MPK1-specific transcription factors that regulate these genes have yet to be determined.

5 SPORE FORMATION

Sporulation occurs in diploid yeast during conditions of nitrogen starvation in the presence of a nonfermentable carbon source. A MAPK gene, SMK1, has been identified as a regulator of spore formation (Krisak *et al.*, 1994). A STE20-like serine/threonine protein kinase, called SPS1, is also required in sporulation (Friesen *et al.*, 1994). Yeast strains mutant in either of these genes are defective in spore wall biosynthesis. Both SMK1 and SPS1 transcripts are expressed in a sporulation-specific manner and are required for the normal expression of late sporulation-specific genes. SPS1 and SMK1 probably represent components in a MAPK module specific to the sporula-

tion process. However, the homologs of MEKK and MKK in this pathway have not yet been identified.

B. *Schizosaccharomyces pombe* (Fission Yeast)

1. PHEROMONE RESPONSE PATHWAY

Sexual differentiation in *S. pombe* is similar in many respects to *S. cerevisiae*; however, there are fundamental disparities. In particular, fission yeast require a mating pheromone as well as nutritional starvation in order to mate. These are respectively mediated by the pheromone response pathway and the stress response MAPK pathway (discussed later). Hence, sexual differentiation involves two distinct MAPK signaling pathways. Another difference is that G protein signaling is transmitted through the G_α subunit in *S. pombe*. Furthermore, Ras is required for the pheromone response, whereas it has no such role in *S. cerevisiae*.

Mating in *S. pombe* occurs between cells of opposite mating type (P or M). Initiation of the pheromone response takes place when P or M pheromones associate with their receptors (Mam2 or Mam3, respectively), stimulating the release of GTP-bound G_α. The G_α subunit, encoded by the *gpa1*$^+$ gene, is required for both mating and sporulation (Obara *et al.*, 1991), although effectors that interact with G_α are not known at present. An essential constituent of the pheromone pathway is Ras1 (Nadin-Davis *et al.*, 1986), which regulates a MAPK module composed of Byr2 (MEKK), Byr1 (MKK), and Spk1 (MAPK) (Nadin Davis and Nasim, 1988, 1990; Wang *et al.*, 1991; Toda *et al.*, 1991). Each component of this MAPK cascade is necessary for the execution of sexual differentiation. Genetic evidence indicates that G_α and Ras1 coordinately stimulate the kinase module, converging at Byr2 (Xu *et al.*, 1994). Ras1 directly binds to the N terminus of Byr2 in a GTP-dependent manner, although this interaction has not been reported to activate Byr2 (Van Aelst *et al.*, 1993; Masuda *et al.*, 1995).

Ras1 also indirectly activates the MAPK module through the STE20 homolog, Shk1/Pak1. Two-hybrid interaction experiments have shown that Ras1·GTP forms a complex with Scd1 (CDC24 homolog), Scd2 (BEM1 homolog), and the small GTPase Cdc42, important for both mating and morphogenesis (Chang *et al.*, 1994). Shk1 binds to Cdc42 both *in vitro* and *in vivo*, and thus is likely to be a Cdc42 effector (Marcus *et al.*, 1995; Ottilie *et al.*, 1995). Shk1 restores mating in *ste20*Δ strains of *S. cerevisiae*, and recombinant Shk1 is able to activate both ERK2 and JNK/SAPK in *Xenopus* oocyte extracts (Polverino *et al.*, 1995), supporting its involvement in MAPK signaling. Another protein essential for sexual differentiation, Ste4, binds the N-terminal regulatory domain of Byr2 at a site distinct from the Ras-1 binding domain (Okazaki *et al.*, 1991; Barr *et al.*, 1996). Ste4 acts upstream of Byr2, and Byr2 requires Ste4 binding for complete function. Therefore, full activation of the MAPK cascade involves multiple inputs at the point of Byr2.

Signaling downstream of Byr2 is conducted through the Byr2–Byr1–Spk1 pathway, which is structurally and functionally homologous to the *S. cerevisiae* STE11–STE7–FUS3/KSS1 module (Neiman *et al.*, 1993). Consistent with homology and complementation data, tyrosine phosphorylation of Spk1 is dependent on Byr1 (Gotoh *et al.*, 1993). The mat1-Pm gene, which controls entry into meiosis, is positively regulated by signaling through the pheromone response pathway (Aono *et al.*, 1994). The transcription factor, Ste11, is a regulator of numerous mating-specific genes, including mat1-Pm; however, it has not been shown to be a substrate of Spk1 (Sugimoto *et al.*, 1991).

2. STRESS/SEXUAL RESPONSE PATHWAY

In *S. pombe*, multiple forms of environmental stress activate a MAPK cascade with components Wik1/Wak1/Wis4 (MEKK), Wis1 (MKK) and Spc1/Sty1/Phh1 (MAPK) (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995a; Kato *et al.*, 1996; Samejima *et al.*, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). Spc1 is phosphorylated and activated by Wis1 in response to hyperosmolarity, starvation, oxidative stress, and heat shock (Millar *et al.*, 1995; Shiozaki and Russell, 1995a; Degols *et al.*, 1996). Like the HOG pathway, the Wik1-Wis1-Spc1 module is regulated by an upstream two-component system (Shieh *et al.*, 1997). Mcs4 is homologous to *S. cerevisiae* SSK1 and functions upstream of Wik1, which activates Wis1 by phosphorylation. Wis1 can also be activated by Win1 in response to osmotic stress independent of Mcs4/Wik1, while Spc1 is activated by heat and oxidative stress independent of Wis1, perhaps involving Pyp1 phosphatase inactivation (Samejima *et al.*, 1997).

This MAPK pathway is regulated by several phosphatases from both the PTPs and PP2C families, in a situation analogous to the HOG pathway. The PTPs are Pyp1 and Pyp2, which are negative regulators of mitosis (Ottilie *et al.*, 1991, 1992; Millar *et al.*, 1992; Hannig *et al.*, 1994). Pyp1 and Pyp2 both form complexes with Spc1 and efficiently inactivate it by direct dephosphorylation of Tyr173 (Shiozaki and Russell, 1995a; Millar *et al.*, 1995). Interestingly, Spc1 activation induces expression of Pyp2, forming a negative feedback loop (Millar *et al.*, 1995; Degols *et al.*, 1996; Wilkinson *et al.*, 1996). The PP2C-related phosphatases, Ptc1, Ptc2, and Ptc3, are also implicated in downregulating the Wis1/Spc1 pathway, and mutants in both Spc1 and Wis1 suppress the lethality of a Δptc1Δptc3 double mutant (Shiozaki *et al.*, 1994; Shiozaki and Russell, 1995b). However, the particular substrate(s) for Ptc has yet to be determined. Dual specificity phosphatases that act on this pathway have not been reported at this time.

Activation of Spc1 is required for the transcription of genes involved in stress responses and sexual development. Examples of stress-responsive genes induced by the Spc1 pathway are glycerol-3-phosphate dehydrogenase (*gpd1*$^+$), trehalose-6-phosphate synthase (*tps1*$^+$), and catalase (λ) (Aiba *et al.*, 1995; Degols *et al.*, 1996; Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). Spc1 is also essential for expression of the Ste11 transcription factor, which regulates sexual differentiation genes in response to nitrogen starvation (Kato *et al.*, 1996; Shiozaki and Russell, 1996). This implicates the Wis1/Spc1 pathway as an essential element in sexual differentiation. Atf1, a transcription factor homologous to mammalian ATF-2 (Takeda *et al.*, 1995), is a likely effector of transcriptional regulation by Spc1. *In vitro*, Atf1 and Spc1 physically associate and Spc1 phosphorylates Atf1. Furthermore, stress-induced expression of *gpd1*$^+$, *tps1*$^+$, catalase, *pyp2*$^+$, and *ste11*$^+$ requires Atf1 (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). However, Atf1 deletion mutants are not defective in mitosis, indicating a branch point in the Wis1/Spc1 pathway between mitotic and stress responses.

In *S. pombe*, the stress response pathway may indirectly stimulate the pheromone response pathway, an example of cross-regulation between MAPK cascades. Transcription of the Byr2 regulator, Ste4, is dependent on Ste11 (Okazaki *et al.*, 1991). Thus, regulation of the pheromone response pathway through Ste4 may occur following Ste11 expression in response to the stress pathway. This may explain the dual requirement for both pheromone factor and nutritional starvation in the mating response.

3. CELL WALL INTEGRITY PATHWAY

A third MAPK isolated in *S. pombe*, called Pmk1/Smp1, is a potential regulator of cell wall integrity (Toda *et al.*, 1996; Zaitsevskaya-Carter and Cooper, 1997). *pmk1Δ* strains have multiple phenotypes including cell wall weakness, unusual cell shape, defective cytokinesis, and altered cation sensitivity. Pmk1 shares 65% identity with *S. cerevisiae* Mpk1, and Mpk1 overexpression is able to rescue *pmk1Δ* phenotypes. However, despite the similarities between these two MAPKs, Pmk1 is not activated by either hypoosmolarity or heat shock nor does it appear as though Pmk1 regulates cell wall integrity in a linear pathway with PKC. *S. pombe* has two known PKCs, *pck1*$^+$ and *pck2*$^+$ (Mazzei *et al.*, 1993; Toda *et al.*, 1993), encoding homologs of the δ, ε, and η isoforms of mammalian PKC. Strains lacking Pck2 have a cell wall defect (Shiozaki and Russell, 1995b), that is more severe than the Pmk1 null strain. However, genetic data argue that Pmk1 and Pck1/2 do not function independently in the regulation of cell wall integrity, but rather in a coordinate manner. One possibility is that Pck1/2 regulate a bifurcated pathway with Pmk1 functioning in one of the branches.

VII. INTRACELLULAR TARGETING AND SPATIAL REGULATION OF MAPK PATHWAY COMPONENTS

A. Signaling Complexes

An important principle that has emerged from studies on kinase cascades is that protein kinases and their targets interact in a way that confers specificity in signaling. Part of this specificity can be attributed to recognition motifs within the active site of each protein kinases. For example, a systematic comparison of different chimeras between ERK2 and p38 MAPK showed that helix C within the N-terminal lobe of the conserved kinase structure is an important determinant for recognition by growth factor vs stress signaling (Brunet and Pouysségur, 1996). Presumably, this domain forms part of the recognition surface between MKKs and MAPKs, as observed in the recognition surface between cyclin A and cdk2 (Jeffrey *et al.*, 1995).

Domains outside the active site may extend the interaction surface between various enzymes and their substrates. For example, stable complexes between STE7 and FUS3 involve interactions between FUS3 and the STE7 N terminus (Bardwell *et al.*, 1996), and a proline-rich domain between subdomains 9 and 10 of MKK1 is important for association between MKK1 and Raf-1 (Papin *et al.*, 1996). Extensive regulatory domains containing conserved interaction motifs are present within several protein kinases, such as Raf-1, MEKKs, PAKs, and multilineage kinases, providing mechanisms to confer multiple selective interactions that augment active site recognition. Complexes formed through such extensive protein–protein interactions would be expected to facilitate signal transmission and quite possibly provide an additional insulating function, preventing cross-regulation between homologous enzymes in growth/differentiation vs stress regulatory pathways. The best example for this is in *S. cerevisiae*, where complex formation of STE5 with STE4, BEM1, STE11, STE7, and/or FUS3 provides a mechanism to facilitate the interaction between several pathway components. However, STE5 appears to be unique to the pheromone response pathway. Homologs have not been found in other systems, and STE5 does not interact with *S. pombe* MEKK or MKK (Byr2, Byr1) nor does it bind to mammalian MEKK, MKK, or Raf (Marcus *et al.*, 1994). Thus, in other cell types, conserved binding motifs within the kinases themselves might provide the scaffolding for interactions that facilitate signaling.

Most of the evidence for stable complexes formed between protein kinases and their substrates is derived from biochemical coprecipitation experiments as well as from two-hybrid interaction screens. Raf-1 and B-Raf form stable complexes with MKK1 and ERKs, mediating the interaction of these kinases with Ras (Moodie *et al.*, 1993; Jelinek *et al.*, 1996; Papin *et al.*, 1995, 1996) and potential interactions with other proteins such as 14-3-3 or KSR.

Raf-1 also interacts with $G_{\beta\gamma}$ subunits and tyrosine kinases Src and Fyn (Cleghon *et al.*, 1994; Pumiglia *et al.*, 1995). Evidence also exists for stable interactions between Ras with MEKK (Russell *et al.*, 1995), Rac-GTP or Cdc42-GTP with PAK20 or MLK-3 (Bagrodia *et al.*, 1995; Teramoto *et al.*, 1996), MLK-3 with JNK1/SAPK1 or MKK3 (Tibbles *et al.*, 1996), and JNKK/SAPK1 with JNK/SAPK (Zanke *et al.*, 1996).

Interactions between MAPKs and their downstream substrates have also been detected biochemically, best exemplified by JNK/SAPK interactions with c-Jun, which are stable enough to enable the detection and purification of JNK/SAPK (Hibi *et al.*, 1993). These interactions are mediated through interactions between a JNK docking site removed from the phosphorylation site on c-Jun and a specificity determination site on JNK/SAPK outside the kinase catalytic cleft (Kallunki *et al.*, 1994,1996). Other substrates that interact with JNK/SAPK include transcription factors ATF and Elk-1 (Bocco *et al.*, 1996; Gupta *et al.*, 1996), and p38 MAPK has been found complexed with MAPKAP kinase-2 and 3pk (McLaughlin *et al.*, 1996; Krump *et al.*, 1997). Substrates that copurify with ERK include MAPKAP kinase-1, c-Myc, and c-Jun (Seimeca *et al.*, 1992; Hsiao *et al.*, 1994; Gupta and Davis, 1994; Bernstein *et al.*, 1994).

B. Nuclear Translocation of MAPK and MKK

Signaling from an extracellular ligand at the plasma membrane requires the migration of at least one of the components in the signaling cascade into the nucleus, where an important end result is transcriptional activation. In some cases, MAPKs fulfill this function, enabling regulation of gene expression through phosphorylation of nuclear transcription factors. The localization of ERK1 and ERK2 is predominantly cytoplasmic in quiescent cells (Chen, R. *et al.*, 1992; Lenormand *et al.*, 1993). However, upon serum or growth factor stimulation, a fraction of cytoplasmic ERK translocates into nuclei (Chen, R. *et al.*, 1992; Traverse *et al.*, 1992; Lenormand *et al.*, 1993; Gonzalez *et al.*, 1993). Translocation occurs rapidly (within 5–30 min, depending on cell type) and typically endures for several hours. Similarly, JNK1/SAPK is both cytoplasmic and nuclear in unstimulated cells, but upon UV irradiation migrates into the nucleus (Cavigelli *et al.*, 1995). Although γ radiation also stimulates JNK1/SAPK activity, it does not appear to cause nuclear translocation (Chen, Y. *et al.*, 1996a). Although a study of endogenous p38 MAPK translocation has yet to be reported, overexpressed p38 MAPK localizes at the cell periphery, in the cytoplasm, and in the nucleus; however, UV irradiation does not lead to its redistribution between cytoplasm and nucleus (Raingeaud *et al.*, 1995). ERK3, unlike the other MAPKs, is constitutively nuclear (Cheng *et al.*, 1996).

Mechanisms for MAPK translocation have yet to be determined. Neither ERK1 nor ERK2 contain nuclear localization or nuclear export signals, and ERKs are below the size limit for passive diffusion through nuclear pores, suggesting that nuclear export may be passive. One possibility is that ERK is retained in the cytoplasm through its interaction with a cytoplasmic localization factor. One candidate for this factor is MKK1, based on data showing a specific interaction of ERK with the N terminus of MKK, cytoplasmic retention of ERK upon expression of MKK, and reduced coimmunoprecipitation of MKK and ERK on cell stimulation (Fukuda *et al.*, 1997). Interestingly, ERK nuclear translocation does not require its activation as nonphosphorylatable mutants ERK $Thr_{183}Ala$, $Tyr_{185}Phe$ or catalytically inactive ERK still translocate in response to serum (Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). However, the rapid uptake of ERK and its nuclear retention against a gradient following stimulation suggest that active import is also likely.

Unlike the ERKs, MKK1 and MKK2 are cytoplasmic before and after growth factor stimulation (Zheng and Guan, 1994b; Moriguchi *et al.*, 1995a). MKK has a short N-terminal, leucine-rich stretch of amino acids (amino acids 32–44) that acts as a strong and autonomous nuclear export signal (Fukuda *et al.*, 1996). Ovalbumin, covalently coupled to a synthetic peptide containing the MKK export sequence, is directed out of the nucleus immediately after microinjection. Thus, a mechanism is in place to export MKK to the cytoplasm, implying that nuclear import of wild-type MKK may occur under physiological conditions. One study has demonstrated serum-dependent nuclear uptake of MKK mutants lacking the export sequence (Jaaro *et al.*, 1997). This raises the question of whether MKK import is required for signaling. For example, MKK and ERK activation may be elevated over several hours following cell stimulation under certain conditions. The existence of high levels of nuclear phosphatases, including dual specificity phosphatases, which target ERK, necessitates a mechanism for maintaining elevated ERK activity in nuclei. Therefore, one possibility is that MKK nuclear uptake may serve as a means for maintaining active nuclear ERK.

VIII. FUTURE DIRECTIONS

Eukaryotic MAP kinase cascades provide excellent examples of signal transduction mechanisms that embody key principles common to many, if not all, signaling pathways. These include regulation of reversible post-translational modification by phosphorylation, sequential activation of multiple signaling components, and involvement of second messengers. Studies

have revealed additional features of these pathways important for signal transmission, such as stable protein complex formation, translocation between intracellular organelles, cross-regulation, and the involvement of homologous kinase modules in parallel pathways.

Many fundamental questions remain for future studies to investigate the mechanisms by which these pathways are regulated as well as the cellular responses that they control. For example, in several cases, the identification of pathway components is incomplete; in particular, many enzymes that mediate the activation of stress-activated kinases by upstream effectors, as well as the sensors for these stress signals, have yet to be identified. We also have little understanding of how these kinase cascades can control such diverse processes as cell transformation, stress responses, and cell differentiation, and why different types of cells respond to the same pathway in different ways. The substrates found so far for MAPKs are likely to be a small fraction of the total number of targets that exist. We have few clear examples of how phosphorylation regulates their function, and we have yet to identify other substrates for upstream kinases that enable these pathways to bifurcate. Finally, little is known of how the events in signaling and cross-regulation are coordinated in order to modulate and tune the cellular responses to these pathways. These kinase cascades are clearly not fabricated from assorted enzymes jumbled together, but require processes of ordered assembly and hierarchal activation to enable the efficient facilitation of signal transduction. The combined use of genetics, molecular biology, and biochemical approaches, together with newly acquired genomic sequences, now set the stage for further exciting research into this highly complex problem.

ACKNOWLEDGMENTS

This work was supported by Grants GM48521 (N.G.A.) and GM18151 (P.S.S.) from the National Institutes of Health. We thank Katheryn Resing, Irene Ota, Loree Kim, and Donna Lonie for many helpful discussions.

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